

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date
14 July 2005 (14.07.2005)

PCT

(10) International Publication Number
WO 2005/062881 A2

(51) International Patent Classification: Not classified William, C. [US/US]; 2225 Lakeridge Road, Grapevine, TX 76051 (US).

(21) International Application Number: PCT/US2004/043092

(74) Agent: PRATT, John, S.; Kilpatrick Stockton, LLC, Suite 2800, 1100 Peachtree Street, Atlanta, GA 30309 (US).

(22) International Filing Date: 24 December 2004 (24.12.2004)

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(25) Filing Language: English

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(26) Publication Language: English

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(30) Priority Data:
60/532,504 24 December 2003 (24.12.2003) US
60/565,371 26 April 2004 (26.04.2004) US
60/592,098 28 July 2004 (28.07.2004) US

(71) Applicants (for all designated States except US): TRANSGENRX, INC. [US/US]; 1755 Wittington Place Drive, Suite 300, Dallas, TX 75234 (US). THE BOARD OF SUPERVISORS OF LOUISIANA STATE UNIVERSITY AND AGRICULTURAL AND MECHANICAL COLLEGE [US/US]; P.O. Box 25055, Baton Rouge, LA 70894 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): COOPER, Richard, K. [US/US]; 622 Woodview Court, Baton Rouge, LA 70810 (US). ENRIGHT, Frederick, M. [US/US]; 535 Woodleigh, Baton Rouge, LA 70810 (US). FIORETTI,

WO 2005/062881 A2

(54) Title: GENE THERAPY USING TRANSPOSON-BASED VECTORS

— G6P PROMOTER ATS polyA IS G6P promoter Insulin polyA IS —

(57) Abstract: Methods and compositions are presented for the administration of transposon based vectors to an animal or human to provide gene therapy to the animal or human.

5 GENE THERAPY USING TRANSPOSON-BASED VECTORS

FIELD OF THE INVENTION

The present invention relates generally to use of transposon-based vectors in the preparation of a medicament useful for providing therapy, including gene therapy, 10 to animals and humans following administration of the medicament. The vectors of the present invention may be directed to specific tissues, organs and cells where a selected gene is stably incorporated and produces proteins, peptides or nucleic acids which have a therapeutic effect in the animal or human.

15 BACKGROUND OF THE INVENTION

Improved gene delivery technologies are needed for the treatment of disease in animals. Many diseases and conditions can be treated with gene-delivery technologies, which provide a gene of interest to an animal suffering from the disease or the condition. An example of such disease is Type 1 diabetes. Type 1 diabetes is 20 an autoimmune disease that ultimately results in destruction of the insulin producing β -cells in the pancreas. Although animals with Type 1 diabetes may be treated adequately with insulin injections or insulin pumps, these therapies are only partially effective. In addition, hyper- and hypoglycemia occurs frequently despite intensive home blood glucose monitoring. Finally, careful dietary constraints are needed to 25 maintain an adequate ratio of calories consumed. Development of gene therapies providing delivery of the insulin gene into the pancreas of diabetic animals could overcome many of these problems and result in improved life expectancy and quality of life.

Several of the prior art gene delivery technologies employed viruses that are 30 associated with potentially undesirable side effects and safety concerns. The majority of current gene-delivery technologies useful for gene therapy rely on virus-based delivery vectors, such as adeno and adeno-associated viruses, retroviruses, and other

viruses, which have been attenuated to no longer replicate. (Kay, M.A., et al. 2001. *Nature Medicine* 7:33-40).

There are multiple problems associated with the use of viral vectors. First, they are not tissue-specific. In fact, a gene therapy trial using adenovirus was recently 5 halted because the vector was present in a patient's sperm (Gene trial to proceed despite fears that therapy could change child's genetic makeup. *The New York Times*, December 23, 2001). Second, viral vectors are likely to be transiently incorporated, which necessitates re-treating a patient at specified time intervals. (Kay, M.A., et al. 2001. *Nature Medicine* 7:33-40). Third, there is a concern that a viral- 10 based vector could revert to its virulent form and cause disease. Fourth, viral-based vectors require a dividing cell for stable integration. Fifth, viral-based vectors indiscriminately integrate into various cells, which can result in undesirable germline integration. Sixth, the required high titers needed to achieve the desired effect have resulted in the death of one patient and they are believed to be responsible for 15 induction of cancer in a separate study. (*Science, News of the Week*, October 4, 2002).

Accordingly, what is needed is a new method to produce transgenic animals and humans with stably incorporated genes, in which the vector containing those genes does not cause disease or other unwanted side effects. There is also a need for 20 DNA constructs that would be stably incorporated into the tissues and cells of animals and humans, including cells in the resting state that are not replicating. There is a further recognized need in the art for DNA constructs capable of delivering genes to specific tissues and cells of animals and humans and for producing proteins in those animals and humans.

25 When incorporating a gene of interest into an animal or human for the production of a desired protein or when incorporating a gene of interest in an animal for the treatment of a disease, it is often desirable to selectively activate incorporated genes using inducible promoters. These inducible promoters are regulated by substances either produced or recognized by the transcription control elements within 30 the cell in which the gene is incorporated. In many instances, control of gene expression is desired in transgenic animals and humans so that incorporated genes are selectively activated at desired times and/or under the influence of specific substances. Accordingly, what is needed is a means to selectively activate genes introduced into the genome of cells of a transgenic animal or human. This can be

taken a step further to cause incorporation to be cell-specific , which prevents widespread gene incorporation throughout the body. This decreases the amount of DNA needed for a treatment, decreases the chance of incorporation in gametes, and targets gene delivery, incorporation, and expression to the desired tissue where the 5 gene is needed to function.

RNAi has been targeted as a tool for several uses including treatment of genetic abnormalities and disease, cancer, and development. There are mainly two types of short RNAs that target complementary messengers in animals: small interfering RNAs and micro-RNAs. Both are produced by the cleavage of double-10 stranded RNA precursors by Dicer, a member of the Rnase III family of double-stranded specific endonucleases, and both guide the RNA-induced silencing complex to cleave specifically RNAs sharing sequence identity with them. RNAi technology can be used in therapeutic approaches to treat disease and various conditions. However, a major drawback to RNAi therapy has been the lack of a reliable delivery 15 method of the short RNA sequences. Most researchers working in the field rely on producing short double stranded RNA (dsRNA) in the laboratory and then delivering these short dsRNAs either by direct injection, electroporation, by complexing with a transfecting reagent, etc. The result is gene silencing, but only as long as the dsRNA remains present in the cell, which generally begins to decrease after about 20 h. In 20 order to obtain lasting therapeutic effects, the RNAi sequence must be expressed long term, preferably under a constitutive promoter. In order to accomplish RNAi expression in a plasmid-based vector and subsequent recognition by RNA induced silencing complex (RISC), the RNA must be double stranded. To obtain dsRNA from a vector, it must be expressed as a short hairpin RNA (shRNA), in which there is a 25 sense strand, a hairpin loop region and an antisense strand (M. Izquierdo. 2004. Short interfering RNAs as a tool for cancer gene therapy. Cancer Gene Therapy pp 1-11; Miyagishi et al. 2004. J Gene Med 6:715-723). The hairpin region allows the antisense strand to loop back and bind to the complimentary sense strand.

30 SUMMARY OF THE INVENTION

The present invention addresses the problems described above by providing new, effective and efficient compositions comprising transposon-based vectors for providing therapy, including gene therapy, to animals and humans. The present invention provides methods of using these compositions for providing therapy to

animals and humans. These transposon-based vectors are used in the preparation of a medicament useful for providing a desired effect to a recipient following administration. Gene therapy includes, but is not limited to, introduction of a gene into an animal using a transposon-based vector. Such genes are called exogenous genes although it is to be understood that these genes may also be found in the recipient animal. These genes may serve a variety of functions in the recipient such as coding for the production of nucleic acids, for example RNA, or coding for the production of proteins and peptides. An advantage of the present invention is that transgenic animals are produced with higher efficiencies than observed in the prior art, including efficient incorporation of large polynucleotide sequences. The present invention facilitates efficient incorporation of the polynucleotide sequences, including the genes of interest, promoters, insertion sequences and poly A, with transfection efficiencies of at least 30%. Transfection efficiencies greater than 30%, 40%, 50%, 60% and 70% have been observed.

Transgenic animals further include but are not limited to avians, fish, amphibians, reptiles, insects, and mammals. It is to be understood that humans are encompassed within the term "animal" and the term "mammal" in the present application. In another embodiment, the animal is a milk-producing animal, including but not limited to bovine, porcine, ovine and equine animals. Transgenic animals include all egg-laying animals and milk-producing animals. In one embodiment, the animal is an avian animal. In another embodiment, the animal is a mammal. Preferred animals may be pets, domestic animals, exotic animals, zoo animals, wild animals or any other type of animal in need of gene therapy. Animals are made transgenic through administration of a composition comprising a transposon-based vector designed for stable incorporation of a gene of interest for production of a desired protein, peptide or nucleic acid, together with an acceptable carrier.

The present invention addresses the problems described above by providing methods and compositions comprising transposon-based vectors for stable incorporation of an exogenous gene into a specific cell or tissue of an animal. It is to be understood that the terms cell-specific and tissue-specific are often used interchangeably by those of ordinary skill in the art. In the present application, cell-specific promoters indicate a promoter that is active in that cell. A promoter may be used to drive the transposase that may or may not drive the desired target protein. For example, a vitellogenin promoter or a glucose-6-phosphatase promoter (both

promoters being found in hepatocytes) may be used to direct incorporation of the gene into the liver, but the promoter driving the target gene/protein, may be constitutive, cell-specific, or be induced by a hormone, antibiotic, etc., that is not specific to a hepatocyte.

5 In one embodiment of the present invention, the transposon-based vectors are designed for cell-specific gene expression, for example, by placing a selected gene under control of a cell-specific promoter, which further increases cell or tissue specificity of expression of the selected gene. In one embodiment of the present invention, the vectors are used for gene therapy of animals or humans, wherein the
10 expression of the selected gene has a therapeutic effect on a cell or a tissue in which it is specifically incorporated, expressed, or both. The expression of the selected gene may also have a therapeutic effect on other cells or tissues, particularly when the expressed protein is secreted from the cell and can access other cells. In another embodiment, the vectors are used to integrate a desired gene into specific cells of an
15 animal for production of biologic agents encoded by the gene of interest.

This invention provides polynucleotide cassettes or vectors containing at least one gene of interest and at least one pro polynucleotide sequences, wherein the at least one gene of interest is operably-linked to a pro nucleotide sequence. Each of the at least one gene of interest encodes a polypeptide. This invention also provides
20 polynucleotide cassettes containing two or more genes of interest and two or more pro polynucleotide sequences, wherein each gene of interest is operably-linked to a pro nucleotide sequence. Each of the genes of interest encodes a polypeptide that forms a part of the multimeric protein. One discovery of the present invention is the use of pro portions of prepro signal sequences to facilitate appropriate processing,
25 expression, and/or formation of multimeric proteins in an individual. Several examples of prepro polynucleotides from which a pro polynucleotide can be derived or be a part of are a cecropin prepro, lysozyme prepro, ovomucin prepro, ovotransferrin prepro, a signal peptide for tumor necrosis factor receptor (SEQ ID NO:1). Signal sequences for protein secretion are readily available through databases
30 such as GenBank or the literature. Signal sequences for protein secretion can be identified by one of ordinary skill in the art, for example through comparison of mRNA to mature protein and identification of the sequence removed from the secreted protein. The prepro or pro polynucleotide can be a cecropin prepro or pro polynucleotide selected from the group consisting of cecropin A1, cecropin A2,

5 cecropin B, cecropin C, cecropin D, cecropin E and cecropin F. In a preferred embodiment, the pro polynucleotide is a cecropin B pro polynucleotide having a sequence shown in SEQ ID NO:2 or SEQ ID NO:3. A preferred prepro polynucleotide is a cecropin B polynucleotide having a sequence shown in SEQ ID NO:4 or SEQ ID NO:5.

10 Another discovery of the present invention is that cecropin prepro sequences facilitate appropriate processing, expression, and/or formation of proteins, including multimeric proteins, in an individual. Accordingly, the present invention includes polynucleotide cassettes containing one or more genes of interest operably-linked to a cecropin prepro sequence. In one embodiment, the polynucleotide cassette contains two or more genes of interest operably-linked to a cecropin prepro sequence. Preferred cecropin prepro polynucleotides are provided in SEQ ID NO:4 and SEQ ID NO:5. The present invention also includes polynucleotide cassettes containing two or more genes of interest operably linked to a cecropin prepro polynucleotide, wherein 15 pro sequences are located between the genes of interest.

These polynucleotide cassettes are administered to an individual for expression of polypeptide sequences and the formation of a protein or a multimeric protein.

20 In another embodiment of the present invention, the gene of interest incorporated into the cell is expressed and the resulting protein or peptide has regulatory properties affecting a function of the cell or tissue in which it is expressed.

In a further embodiment, the gene of interest incorporated into the cell is expressed, secreted and affects another cell.

25 In another embodiment of the present invention, the gene of interest incorporated into the cell is expressed as an inhibitory molecule, such as an RNAi may regulate the expression or overexpression of another substance.

30 Cell or tissue-specific expression of a gene of interest may be particularly advantageous because of the possible toxic or otherwise potentially negative effects of the gene of interest if it were expressed in an undesirable location. Control of expression in the desired cell or tissue is achieved by operably linking the gene of interest to a cell or tissue specific regulator.

In yet another embodiment of the present invention, the expression of the exogenous gene is blocked unless an animal is at a selected stage in its life cycle. For example, expression of an exogenous gene may not be desirable until an animal

reaches puberty. In this embodiment, the expression of the exogenous gene is controlled by a promoter, which is activated specifically at the desired stage in the life of the animal. Alternatively, expression of an exogenous gene may not be desirable until a disease process begins or at a time when a substance, such as a hormone or an 5 inflammatory molecule, is found in undesirable levels.

In yet another embodiment of the present invention, the expression of the exogenous gene is blocked unless an animal produces a substance. For example, expression of an exogenous gene may not be desirable until an animal begins producing cancer-related molecules. In this embodiment, the expression of the 10 exogenous gene is controlled by a promoter, which is activated specifically by the cancer-related molecules. Such gene therapy fights the disease process at very early stages.

An additional advantage of the present invention is that a disease or a condition can be treated in a specific organ or tissue of an animal without risk of 15 making other organs or tissues transgenic. This is particularly useful when concerns exist about passing a transgene to the progeny of the transgenic animal, or contaminating the environment with the transgene shed by the animal. The methods and compositions of the present invention are particularly advantageous in applications where germline integration of exogenous DNA is undesirable.

20 The compositions of the present invention may be introduced into an animal through any route of administration that serves to deliver the composition to the desired organs, tissues and cells. Such routes of administration include, but are not limited to, oral and parenteral routes such as intravascular, intravenous, intraarterial, intracardiac, intraperitoneal, intramuscular, anal, intracerebrovascular, 25 intracerebroventricular, cutaneous, intradermal, subcutaneous, transdermal, into any duct system, into any cavity or space, such as the abdominopelvic, pleural, pericardial, peritoneal cavities or spaces, intrathecal, or into the respiratory system, the urinary system, the gastrointestinal system, the nervous system, the lymphatic system, the immune system, the reproductive system and the endocrine system.

30 Administration of the transposon based vectors into the cardiovascular system achieves rapid distribution throughout the animal to reach target tissues and cells receiving blood supply. Such administration may be into any chamber of the heart, for example into the left ventricle, the right ventricle, or the atrial chambers, for rapid distribution into the systemic circulation or the pulmonary circulation. The vectors

may be administered into selected vessels, such as the cardiac vessels, into the aorta or into a selected vessel leading to a targeted group of cells, a tissue, an organ or a tumor. Administration into the left side of the heart may target the systemic circulation through the aorta and any of its branches, including but not limited to the 5 coronary vessels, the ovarian or testicular arteries, the renal arteries, the arteries supplying the gastrointestinal and pelvic tissues, including the celiac, cranial mesenteric and caudal mesenteric vessels and their branches, the common iliac arteries and their branches to the pelvic organs, the gastrointestinal system and the lower extremity, the carotid, brachiocephalic and subclavian arteries. It is to be 10 understood that the specific names of blood vessels change with the species under consideration and are known to one of ordinary skill in the art. Administration into the left ventricle or ascending aorta supplies any of the tissues receiving blood supply from the aorta and its branches, including but not limited to the testes, ovary, oviduct, and liver. Administration may occur through any means, for example by injection 15 into the left ventricle, or by administration through a cannula or needle introduced into the left atrium, left ventricle, aorta or a branch thereof.

The compositions of the present invention may be administered to a reproductive organ including, but not limited to, an oviduct, an ovary, the testes, seminal vesicle, any accessory organ, or into the duct system of the mammary gland. 20 The compositions of the present invention may be administered to a reproductive organ of an animal through the cloaca. The compositions of the present invention may be directly administered to an organ or can be administered to an artery or vein leading to the organ. A transfection reagent is optionally added to the composition before administration.

25 The transposon-based vectors of the present invention include a transposase, operably-linked to a first promoter, and a coding sequence for a protein or peptide of interest operably-linked to a second promoter, wherein the coding sequence for the protein or peptide of interest and its operably-linked promoter are flanked by transposase insertion sequences recognized by the transposase. The transposon-based 30 vector also includes the following characteristics: a) one or more modified Kozak sequences at the 3' end of the first promoter to enhance expression of the transposase; b) modifications of the codons for the first several N-terminal amino acids of the transposase, wherein the nucleotide at the third base position of each codon is changed to an A or a T without changing the corresponding amino acid; c) addition of

one or more stop codons to enhance the termination of transposase synthesis; and/or,
d) addition of an effective polyA sequence operably-linked to the transposase to
further enhance expression of the transposase gene. In some embodiments, the
effective polyA sequence is an avian optimized polyA sequence.

5 The present invention also provides for tissue-specific incorporation and/or
expression of a gene of interest. Tissue-specific stable incorporation of a gene of
interest may be achieved by placing the transposase gene under the control of a tissue-
specific promoter, whereas tissue-specific expression of a gene of interest may be
achieved by placing the gene of interest under the control of a tissue-specific
10 promoter. Such tissues include all tissues within the body, for example, connective
tissue, muscle, bone, lymphoid tissue, and nervous tissue. In some embodiments, the
gene of interest is transcribed under the influence of an ovalbumin, or other oviduct
specific, promoter. In other embodiments, promoters may be specific for cells in
another organ including but not limited to the liver, brain, mammary gland, any
15 endocrine organ, and thymus.

The present invention also provides for cell-specific incorporation and/or
expression of a gene of interest. Cell-specific incorporation of a gene of interest may
be achieved by placing the transposase gene under the control of a cell-specific
promoter, whereas tissue-specific expression of a gene of interest may be achieved by
20 placing the gene of interest under the control of a cell-specific promoter. Such
promoters may include promoters specific for cells such as neurons, glia, hepatocytes,
epithelial cells, cells of the immune system, fibroblasts, chondrocytes, synovial cells,
osteoblasts, osteocytes, osteoclasts, muscle cells (including striated, smooth and
cardiac muscle cells), granulocytes, lymphocytes, T lymphocytes, B-lymphocytes,
25 thymocytes, germ bells, blast cells, cancerous cells, endocrine cells, white blood cells,
pancreatic islet cells, acinar cells, splenocytes, follicular cells, and so on. Cell
specific promoters are known to one of ordinary skill in the art.

The present invention advantageously produces a high number of transgenic
animals having a gene of interest stably incorporated. In some embodiments wherein
30 the transposon-based vector is administered to the ovary or the testes, these transgenic
animals successfully pass the desired gene to their progeny. Accordingly, the present
invention can be used to obtain transgenic animals having the gene of interest
incorporated into the germline through transfection of the ovary or testes. These
transgenic animals of the present invention produce large amounts of a desired

molecule encoded by the transgene. Such germline transmission can produce generations of animals containing the desired gene. Such a gene may be useful in providing gene therapy to animals known to be susceptible to specific conditions, for example an immune deficiency or arthritis.

5 Gene therapy of cells other than germline cells may also be achieved by introducing the transposon-based vectors to these cells for stable incorporation of exogenous genes.

Any desired gene may be incorporated into the novel transposon-based vectors of the present invention in order to synthesize a desired molecule in the transgenic 10 animals to provide gene therapy. Proteins, peptides and nucleic acids are preferred desired molecules to be produced by the transgenic animals of the present invention. In order to provide gene therapy to an animal, the gene desired to produce a desired molecule in the transgenic animal is selected and inserted into the transposon-based vectors of the present invention.

15 Nucleic acids may be made by the transgenic animals. Such nucleic acids include, but are not limited to, single stranded DNA, RNA, antisense nucleic acids, siRNA, and polynucleotide strands that affect cellular function. Some of these nucleic acids may affect cellular function by modulating transcription of a gene, for example by inhibiting the function of the gene which may be producing undesirable 20 effects such as inappropriate amounts of molecules or molecules that may be deleterious to the animal.

25 Genes may be regulated by regulating a specific transcription factor. Cystic fibrosis, for example, is the result of a mutant protein. Even if RNAi is used, the cell is still synthesizing mutant RNA. However, if the transcription factor allowing expression of the mutant protein is blocked, then the mutant gene is shut down and the normal gene can be expressed under a different promoter.

A wide range of recombinant peptides and proteins can be produced in animals receiving the gene therapy of the present invention. Enzymes, hormones, 30 antibodies, growth factors, serum proteins, commodity proteins, fusion proteins, fusion peptides, biological response modifiers, cytokines, chemoattractants, chemorepellents, receptor agonists, receptor antagonists, peptides and designed proteins may all be made through practice of the present invention.

Accordingly, it is an object of the present invention to provide novel transposon-based vectors useful in providing gene therapy to an animal.

It is an object of the present invention to provide novel transposon-based vectors for use in the preparation of a medicament useful in providing gene therapy to an animal or human.

5 It is another object of the present invention to provide novel transposon-based vectors that encode for the production of desired proteins or peptides in cells.

Yet another object of the present invention to provide novel transposon-based vectors that encode for the production of desired nucleic acids in cells.

10 It is a further object of the present invention to provide methods for cell and tissue specific incorporation of transposon-based DNA constructs comprising targeting a selected gene to a specific cell or tissue of an animal.

It is yet another object of the present invention to provide methods for cell and tissue specific expression of transposon-based DNA constructs comprising designing a DNA construct with cell specific promoters that enhance stable incorporation of the selected gene by the transposase and expressing the selected gene in the cell.

15 15 It is an object of the present invention to provide gene therapy for generations through germ line administration of a transposon-based vector.

Another object of the present invention is to provide gene therapy in animals through non germ line administration of a transposon-based vector.

20 It is further an object of the present invention to provide a method to produce transgenic animals through intraovarian or intratesticular administration of a transposon-based vector that are capable of producing transgenic progeny.

It is further an object of the present invention to provide a method to produce transgenic animals through cardiovascular administration of a transposon-based vector.

25 25 Another object of the present invention is to provide gene therapy in animals through administration of a transposon-based vector, wherein the animals produce desired proteins, peptides or nucleic acids.

30 Yet another object of the present invention is to provide gene therapy in animals through administration of a transposon-based vector, wherein the animals produce desired proteins or peptides that are recognized by receptors on target cells.

Still another object of the present invention is to provide gene therapy in animals through administration of a transposon-based vector, wherein the animals produce desired fusion proteins or fusion peptides, a portion of which are recognized

by receptors on target cells, in order to deliver the other protein or peptide component of the fusion protein or fusion peptide to the cell to induce a biological response.

Yet another object of the present invention is to provide a method for gene therapy of animals through administration of transposon-based vectors comprising 5 tissue specific promoters and a gene of interest to facilitate tissue specific incorporation and expression of a gene of interest to produce a desired protein, peptide or nucleic acid.

Another object of the present invention is to provide a method for gene therapy of animals through administration of transposon-based vectors comprising 10 cell specific promoters and a gene of interest to facilitate cell specific incorporation and expression of a gene of interest to produce a desired protein, peptide or nucleic acid.

Still another object of the present invention is to provide a method for gene therapy of animals through administration of transposon-based vectors comprising 15 cell specific promoters and a gene of interest to facilitate cell specific incorporation and expression of a gene of interest to produce a desired protein, peptide or nucleic acid, wherein the desired protein, peptide or nucleic acid has a desired biological effect in the animal.

Another object of the present invention is to provide transgenic animals that 20 contain a stably incorporated transgene.

An advantage of the present invention is that transgenic animals are produced with higher efficiencies than observed in the prior art.

Another advantage of the present invention is that transgenic animals are produced with higher efficiencies than observed in the prior art, including large 25 transgenes.

Another advantage of the present invention is that these transgenic animals possess high copy numbers of the transgene.

Another advantage of the present invention is that the transgenic animals produce large amounts of desired molecules encoded by the transgene.

30 Still another advantage of the present invention is that desired molecules are produced by the transgenic animals much more efficiently and economically than prior art methods, thereby providing a means for efficient gene therapy.

Yet another advantage of the present invention is that the desired proteins and peptides are produced rapidly after making animals transgenic through introduction of the vectors of the present invention.

These and other objects, features and advantages of the present invention will 5 become apparent after a review of the following detailed description of the disclosed embodiments and claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts schematically a transposon-based vector containing a 10 transposase operably linked to a first promoter and a gene of interest and poly A operably-linked to a second promoter, wherein the gene of interest and its operably-linked promoter are flanked by insertion sequences (IS) recognized by the transposase. "Pro" designates a promoter. In this and subsequent figures, the size of the actual nucleotide sequence is not necessarily proportionate to the box representing 15 that sequence.

Figure 2 depicts schematically a transposon-based vector for targeting deposition of a polypeptide in an egg white wherein Ov pro is the ovalbumin promoter, Ov protein is the ovalbumin protein and PolyA is a polyadenylation sequence. The TAG sequence includes a spacer sequence, the gp41 hairpin loop from 20 HIV I and a protease cleavage site.

Figure 3 depicts schematically a transposon-based vector for targeting deposition of a polypeptide in an egg white wherein Ovo pro is the ovomucoid promoter and Ovo SS is the ovomucoid signal sequence. The TAG sequence includes a spacer, the gp41 hairpin loop from HIV I and a protease cleavage site.

Figure 4 depicts schematically a transposon based-vector for expression of an 25 RNAi molecule. "Tet_r pro" indicates a tetracycline inducible promoter whereas "pro" indicates the pro portion of a prepro sequence as described herein. "Ovgen" indicates approximately 60 base pairs of an ovalbumin gene, "Ovotrans" indicates approximately 60 base pairs of an ovotransferring gene and "Ovomucin" indicates 30 approximately 60 base pairs of an ovomucin gene.

Figure 5 is a schematic of a vector for stable transformation of liver cells for production of insulin. This vector provides for stable transformation of hepatocytes and allows the production of insulin in response to blood glucose levels.

Figure 6 depicts schematically a transposon-based vector targeted to hepatocytes for use in gene therapy for diabetes. A glucose-6-phosphatase (G-6-P) promoter is placed upstream of the transposase (ATS) and another G-6-P promoter is upstream of the proinsulin gene and the polyadenylation sequence (PolyA). Insertion sequences (IS) recognized by the transposase flank the G-6-P promoter, proinsulin gene and PolyA.

Figure 7 depicts schematically a transposon-based vector targeted to hepatocytes for use in gene therapy for production of growth hormone and treatment of growth hormone deficiencies. A glucose-6-phosphatase (G-6-P) promoter is placed upstream of the transposase (ATS) and an albumin promoter is upstream of the human growth hormone (hGH) gene and the polyadenylation sequence (PolyA). Insertion sequences (IS) recognized by the transposase flank the albumin promoter, GH gene and PolyA.

Figure 8 depicts schematically a transposon-based vector targeted to respiratory epithelial cells for use in gene therapy for treatment of cystic fibrosis. A ciliated cell-specific promoter (FOXJ1) is placed upstream of the transposase (ATS), normal CFTR gene and the polyadenylation sequence (PolyA). Insertion sequences (IS) recognized by the transposase flank the normal cystic fibrosis transmembrane conductance regulator gene (CFTR) and PolyA.

Figure 9 depicts schematically a transposon-based vector targeted to cancer cells for use in gene therapy for treatment of cancer. Human telomerase reverse transcriptase/human surfactant protein A1 (HTERT/hSPA1) is placed upstream of the transposase (ATS). A SV40p promoter is placed upstream of the a gene for cholera toxin and the polyadenylation sequence (PolyA). Insertion sequences (IS) recognized by the transposase flank the SV40p promoter , cholera toxin gene and PolyA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new, effective and efficient method of providing gene therapy to animals through administration of a composition comprising a transposon-based vector designed for incorporation of a gene of interest and production of a desired molecule. These transposon-based vectors are used in the preparation of a medicament for administration to an animal or human to provide a beneficial effect in the recipient through production of a desired molecule. The present invention facilitates efficient incorporation of the polynucleotide sequences,

including the genes of interest, promoters, insertion sequences and poly A, with transfection efficiencies of at least 30%. Transfection efficiencies greater than 30%, 40%, 50%, 60% and 70% have been observed. Desired molecules encoded by genes of interest include, but are not limited to, nucleic acids, proteins and peptides.

5 Proteins include multimeric proteins. Multimeric proteins include associated multimeric proteins (two or more associated polypeptides) and multivalent multimeric proteins (a single polypeptide encoded by more than one gene of interest). Expression and/or formation of the multimeric protein in the individual is achieved by administering a polynucleotide cassette containing the genes of interest to the

10 individual. The polynucleotide cassette may additionally contain one or more pro sequences, prepro sequences, cecropin prepro sequences, and/or cleavage site sequences. In a preferred embodiment, the polynucleotide cassette is administered through the vascular system. Nucleic acids that may be produced in transfected cells include single stranded DNA, RNA, antisense nucleic acids, siRNA, and

15 polynucleotide strands that affect cellular function.

Definitions

It is to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for

20 example, reference to "a cell" can mean that at least one cell can be utilized.

The term "animal" includes a human in the present application.

The term "protein" includes multimeric protein" as described in PCT US03/41261. Multimeric proteins include associated multimeric proteins (two or more associated polypeptides) and multivalent multimeric proteins (a single

25 polypeptide encoded by more than one gene of interest).

The term "nucleic acid" includes double stranded DNA, single stranded DNA, RNA, antisense nucleic acids, siRNA, and polynucleotide strands.

The term "antibody" is used interchangeably with the term "immunoglobulin" and is defined herein as a protein synthesized by an animal or a cell of the immune

30 system in response to the presence of a foreign substance commonly referred to as an "antigen" or an "immunogen". The term antibody includes fragments of antibodies. Antibodies are characterized by specific affinity to a site on the antigen, wherein the site is referred to an "antigenic determinant" or an "epitope". Antigens can be naturally occurring or artificially engineered. Artificially engineered antigens

include, but are not limited to, small molecules, such as small peptides, attached to haptens such as macromolecules, for example proteins, nucleic acids, or polysaccharides. Artificially designed or engineered variants of naturally occurring antibodies and artificially designed or engineered antibodies not occurring in nature 5 are all included in the current definition. Such variants include conservatively substituted amino acids and other forms of substitution as described in the section concerning proteins and polypeptides.

The present invention provides novel transposon-based vectors and their use for specific and stable incorporation of a gene of interest into a specific cell to stably 10 incorporate the gene.

The term "gene" is defined herein to include a coding region for a protein, peptide or polypeptide.

The term "transgenic animal" refers to an animal having at least a portion of the transposon-based vector DNA is incorporated into its DNA. While a transgenic 15 animal includes an animal wherein the transposon-based vector DNA is incorporated into the germline DNA, a transgenic animal also includes an animal having DNA in one or more cells that contain a portion of the transposon-based vector DNA for any period of time. In a preferred embodiment, a portion of the transposon-based vector comprises a gene of interest. More preferably, the gene of interest is incorporated into 20 the animal's DNA for a period of at least a few days, preferably the reproductive life of the animal, and preferably the life of the animal.

The term "vector" is used interchangeably with the terms "construct", "DNA construct", "genetic construct", and "polynucleotide cassette" to denote synthetic nucleotide sequences used for manipulation of genetic material, including but not 25 limited to cloning, subcloning, sequencing, or introduction of exogenous genetic material into cells, tissues or organisms, such as animals. It is understood by one skilled in the art that vectors may contain synthetic DNA sequences, naturally occurring DNA sequences, or both. The vectors of the present invention are transposon-based vectors as described herein.

30 When referring to two nucleotide sequences, one being a regulatory sequence, the term "operably-linked" is defined herein to mean that the two sequences are associated in a manner that allows the regulatory sequence to affect expression of the other nucleotide sequence. It is not required that the operably-linked sequences be directly adjacent to one another with no intervening sequence(s).

The term "regulatory sequence" is defined herein as including promoters, enhancers and other expression control elements such as polyadenylation sequences, matrix attachment sites, insulator regions for expression of multiple genes on a single construct, ribosome entry/attachment sites, introns that are able to enhance 5 expression, and silencers. Promoters may be cell specific or tissue specific to facilitate expression in a desired target.

Transposon-Based Vectors

While not wanting to be bound by the following statement, it is believed that 10 the nature of the DNA construct is an important factor in successfully providing gene therapy to animals. The "standard" types of plasmid and viral vectors that have previously been almost universally used for transgenic work in all species have low efficiencies and may constitute a major reason for the low rates of transformation previously observed. The DNA (or RNA) constructs previously used often do not 15 integrate into the host DNA, or integrate only at low frequencies. Other factors may have also played a part, such as poor entry of the vector into target cells. The present invention provides transposon-based vectors that can be administered to an animal that overcome the prior art problems relating to low transgene integration frequencies. In the present invention integration frequencies greater than 30%, 40%, 50%, 60% 20 and also 70% are often obtained for vectors about 10 kb or less. In some cases, depending on the route of administration, integration frequencies of over 70%, 80% and even 90% are observed. If the vector is over 15 kb, then transfection rates of over 30% and over 40% are feasible. Two preferred transposon-based vectors of the 25 present invention in which a transposase, gene of interest and other polynucleotide sequences may be introduced are termed pTnMCS (SEQ ID NO:6) and timed (SEQ ID NO:7).

The transposon-based vectors of the present invention produce integration frequencies an order of magnitude greater than has been achieved with previous vectors. More specifically, intratesticular injections performed with a prior art 30 transposon-based vector (described in U.S. Patent No. 5,719,055) resulted in 41% sperm positive roosters whereas intratesticular injections performed with the novel transposon-based vectors of the present invention resulted in 77% sperm positive roosters. Actual frequencies of integration were estimated by either or both

comparative strength of the PCR signal from the sperm and histological evaluation of the testes and sperm by quantitative PCR.

The transposon-based vectors of the present invention include a transposase gene operably-linked to a first promoter, and a coding sequence for a desired protein or peptide operably-linked to a second promoter, wherein the coding sequence for the desired protein or peptide and its operably-linked promoter are flanked by transposase insertion sequences recognized by the transposase. The transposon-based vector also includes one or more of the following characteristics: a) one or more modified Kozak sequences comprising ACCATG (SEQ ID NO:8) at the 3' end of the first promoter to enhance expression of the transposase; b) modifications of the codons for the first several N-terminal amino acids of the transposase, wherein the third base of each codon was changed to an A or a T without changing the corresponding amino acid; c) addition of one or more stop codons to enhance the termination of transposase synthesis; and/or, d) addition of an effective polyA sequence operably-linked to the transposase to further enhance expression of the transposase gene. The transposon-based vector may additionally or alternatively include one or more of the following Kozak sequences at the 3' end of any promoter, including the promoter operably-linked to the transposase: ACCATGG (SEQ ID NO:9), AAGATGT (SEQ ID NO:10), ACGATGA (SEQ ID NO:11), AAGATGG (SEQ ID NO:12), GACATGA (SEQ ID NO:13), ACCATGA (SEQ ID NO:14), and ACCATGA (SEQ ID NO:15), ACCATGT (SEQ ID NO:16). In another embodiment, the transposon-based vector comprises an avian optimized polyA sequence and does not comprise a modified Kozak sequence.

Figure 1 shows a schematic representation of several components of the transposon-based vector. The present invention further includes vectors containing more than one gene of interest, wherein a second or subsequent gene of interest is operably-linked to the second promoter or to a different promoter. It is also to be understood that the transposon-based vectors shown in the Figures are representative of the present invention and that the order of the vector elements may be different than that shown in the Figures, that the elements may be present in various orientations, and that the vectors may contain additional elements not shown in the Figures.

Transposases and Insertion Sequences

In a further embodiment of the present invention, the transposase found in the transposase-based vector is an altered target site (ATS) transposase and the insertion sequences are those recognized by the ATS transposase. However, the transposase located in the transposase-based vectors is not limited to a modified ATS transposase and can be derived from any transposase. Transposases known in the prior art include those found in AC7, Tn5SEQ1, Tn916, Tn951, Tn1721, Tn 2410, Tn1681, Tn1, Tn2, Tn3, Tn4, Tn5, Tn6, Tn9, Tn10, Tn30, Tn101, Tn903, Tn501, Tn1000 ($\gamma\delta$), Tn1681, Tn2901, AC transposons, Mp transposons, Spm transposons, En transposons, Dotted transposons, Mu transposons, Ds transposons, dSpm transposons and I transposons. According to the present invention, these transposases and their regulatory sequences are modified for improved functioning as follows: a) the addition one or more modified Kozak sequences comprising ACCATG (SEQ ID NO:8) at the 3' end of the promoter operably-linked to the transposase; b) a change of the codons for the first several amino acids of the transposase, wherein the third base of each codon was changed to an A or a T without changing the corresponding amino acid; c) the addition of one or more stop codons to enhance the termination of transposase synthesis; and/or, d) the addition of an effective polyA sequence operably-linked to the transposase to further enhance expression of the transposase gene.

Although not wanting to be bound by the following statement, it is believed that the modifications of the first several N-terminal codons of the transposase gene increase transcription of the transposase gene, in part, by increasing strand dissociation. It is preferable that between approximately 1 and 20, more preferably 3 and 15, and most preferably between 4 and 12 of the first N-terminal codons of the transposase are modified such that the third base of each codon is changed to an A or a T without changing the encoded amino acid. In one embodiment, the first ten N-terminal codons of the transposase gene are modified in this manner. It is also preferred that the transposase contain mutations that make it less specific for preferred insertion sites and thus increases the rate of transgene insertion as discussed in U.S. Patent No. 5,719,055.

In some embodiments, the transposon-based vectors are optimized for expression in a particular host by changing the methylation patterns of the vector

DNA. For example, prokaryotic methylation may be reduced by using a methylation deficient organism for production of the transposon-based vector. The transposon-based vectors may also be methylated to resemble eukaryotic DNA for expression in a eukaryotic host.

5 Transposases and insertion sequences from other analogous eukaryotic transposon-based vectors that can also be modified and used are, for example, the Drosophila P element derived vectors disclosed in U.S. Patent No. 6,291,243; the Drosophila mariner element described in Sherman et al. (1998); or the sleeping beauty transposon. See also Hackett et al. (1999); D. Lampe et al., 1999. Proc. Natl. Acad. 10 Sci. USA, 96:11428-11433; S. Fischer et al., 2001. Proc. Natl. Acad. Sci. USA, 98:6759-6764; L. Zagoraiou et al., 2001. Proc. Natl. Acad. Sci. USA, 98:11474-11478; and D. Berg et al. (Eds.), Mobile DNA, Amer. Soc. Microbiol. (Washington, D.C., 1989). However, it should be noted that bacterial transposon-based elements are preferred, as there is less likelihood that a eukaryotic transposase in the recipient 15 species will recognize prokaryotic insertion sequences bracketing the transgene.

Many transposases recognize different insertion sequences, and therefore, it is to be understood that a transposase-based vector will contain insertion sequences recognized by the particular transposase also found in the transposase-based vector. In a preferred embodiment of the invention, the insertion sequences have been 20 shortened to about 70 base pairs in length as compared to those found in wild-type transposons that typically contain insertion sequences of well over 100 base pairs.

While the examples provided below incorporate a "cut and insert" Tn10 based vector that is destroyed following the insertion event, the present invention also encompasses the use of a "rolling replication" type transposon-based vector. Use of a 25 rolling replication type transposon allows multiple copies of the transposon/transgene to be made from a single transgene construct and the copies inserted. This type of transposon-based system thereby provides for insertion of multiple copies of a transgene into a single genome. A rolling replication type transposon-based vector may be preferred when the promoter operably-linked to gene of interest is endogenous 30 to the host cell and present in a high copy number or highly expressed. However, use of a rolling replication system may require tight control to limit the insertion events to non-lethal levels. Tn1, Tn2, Tn3, Tn4, Tn5, Tn9, Tn21, Tn501, Tn551, Tn951, Tn1721, Tn2410 and Tn2603 are examples of a rolling replication type transposon, although Tn5 could be both a rolling replication and a cut and insert type transposon.

Stop Codons and PolyA Sequences

In one embodiment, the transposon-based vector contains two stop codons operably-linked to the transposase and/or to the gene of interest. In an alternate 5 embodiment, one stop codon of UAA or UGA is operably linked to the transposase and/or to the gene of interest. While not wanting to be bound by the following statement, it is thought that the stop codon UAG is less effective in translation termination and is therefore less desirable in the constructs described herein.

As used herein an "effective polyA sequence" refers to either a synthetic or 10 non-synthetic sequence that contains multiple and sequential nucleotides containing an adenine base (an A polynucleotide string) and that increases expression of the gene to which it is operably-linked. A polyA sequence may be operably-linked to any gene in the transposon-based vector including, but not limited to, a transposase gene and a gene of interest. A preferred polyA sequence is optimized for use in the host animal 15 or human and for the desired end product.

The goal is to use a poly A that gives a similar level of expression as the gene being replaced, or the desired result. With siRNA for example, only a few copies of the RNAi sequence are required, so the mRNA may not have to be extremely stable, and in fact may be detrimental or just a waste of energy for the cell (See Zhang et al., 20 Nucleic Acids Research, database issue, Vol. 33:D116-D120 2005).

In one embodiment, the polyA sequence is optimized for use in an avian species and more specifically, a chicken. An avian optimized polyA sequence generally contains a minimum of 40 base pairs, preferably between approximately 40 and several hundred base pairs, and more preferably approximately 75 base pairs that 25 precede the A polynucleotide string and thereby separate the stop codon from the A polynucleotide string. In one embodiment of the present invention, the polyA sequence comprises a conalbumin polyA sequence as provided in SEQ ID NO:17 and as taken from GenBank accession # Y00407, base pairs 10651-11058. In another embodiment, the polyA sequence comprises a synthetic polynucleotide sequence 30 shown in SEQ ID NO:18. In yet another embodiment, the polyA sequence comprises an avian optimized polyA sequence provided in SEQ ID NO:19. A chicken optimized polyA sequence may also have a reduced amount of CT repeats as compared to a synthetic polyA sequence.

It is a surprising discovery of the present invention that such an avian optimized poly A sequence increases expression of a polynucleotide to which it is operably-linked in an avian as compared to a non-avian optimized polyA sequence. It is to be understood that polyA sequences may be optimized for other classes of 5 animals, such as mammals, and used in the transposon-based vectors of the present invention to provide gene therapy. Accordingly, the present invention includes methods of or increasing incorporation of a gene of interest wherein the gene of interest resides in a transposon-based vector containing a transposase gene and wherein the transposase gene is operably linked to an avian optimized polyA 10 sequence. The present invention also includes methods of increasing expression of a gene of interest in an avian that includes administering a gene of interest to the avian, wherein the gene of interest is operably-linked to an avian optimized polyA sequence. An avian optimized polyA nucleotide string is defined herein as a polynucleotide containing an A polynucleotide string and a minimum of 40 base pairs, preferably 15 between approximately 40 and several hundred base pairs, and more preferably approximately 75 base pairs that precede the A polynucleotide string. The present invention further provides transposon-based vectors containing a gene of interest or transposase gene operably linked to an avian optimized polyA sequence.

20 Promoters and Enhancers

The first promoter operably-linked to the transposase gene and the second promoter operably-linked to the gene of interest can be a constitutive promoter or an inducible promoter. Constitutive promoters include, but are not limited to, immediate early cytomegalovirus (CMV) promoter, herpes simplex virus 1 (HSV1) immediate 25 early promoter, SV40 promoter, lysozyme promoter, early and late CMV promoters, early and late HSV promoters, β -actin promoter, tubulin promoter, Rous-Sarcoma virus (RSV) promoter, and heat-shock protein (HSP) promoter. Inducible promoters include tissue-specific promoters, developmentally-regulated promoters, and chemically inducible promoters. Examples of tissue-specific promoters include the 30 glucose-6-phosphatase (G6P) promoter, vitellogenin promoter, ovalbumin promoter, ovomucoid promoter, conalbumin promoter, ovotransferrin promoter, prolactin promoter, kidney uromodulin promoter, and placental lactogen promoter. In one embodiment, the vitellogenin promoter includes a polynucleotide sequence of SEQ ID

NO:20. The G6P promoter sequence may be deduced from a rat G6P gene untranslated upstream region provided in GenBank accession number U57552.1. Examples of developmentally-regulated promoters include the homeobox promoters and several hormone induced promoters. Examples of chemically inducible 5 promoters include reproductive hormone induced promoters and antibiotic inducible promoters such as the tetracycline inducible promoter and the zinc-inducible metallothioneine promoter.

Other inducible promoter systems include the Lac operator repressor system inducible by IPTG (isopropyl beta-D-thiogalactoside) (Cronin, A. et al. 2001. Genes and Development, v. 15), ecdysone-based inducible systems (Hoppe, U. C. et al. 2000. Mol. Ther. 1:159-164); estrogen-based inducible systems (Braselmann, S. et al. 1993. Proc. Natl. Acad. Sci. 90:1657-1661); progesterone-based inducible systems using a chimeric regulator, GLVP, which is a hybrid protein consisting of the GAL4 binding domain and the herpes simplex virus transcriptional activation domain, VP16, 15 and a truncated form of the human progesterone receptor that retains the ability to bind ligand and can be turned on by RU486 (Wang, et al. 1994. Proc. Natl. Acad. Sci. 91:8180-8184); CID-based inducible systems using chemical inducers of dimerization (CIDs) to regulate gene expression, such as a system wherein rapamycin induces dimerization of the cellular proteins FKBP12 and FRAP (Belshaw, P. J. et al. 1996. J. 20 Chem. Biol. 3:731-738; Fan, L. et al. 1999. Hum. Gene Ther. 10:2273-2285; Shariat, S.F. et al. 2001. Cancer Res. 61:2562-2571; Spencer, D.M. 1996. Curr. Biol. 6:839-847). Chemical substances that activate the chemically inducible promoters can be administered to the animal containing the transgene of interest via any method known to those of skill in the art.

25 Other examples of cell-specific and constitutive promoters include but are not limited to smooth-muscle SM22 promoter, including chimeric SM22alpha/telokin promoters (Hoggatt A.M. et al., 2002. Circ Res. 91(12):1151-9); ubiquitin C promoter (Biochim Biophys Acta, 2003. Jan. 3;1625(1):52-63); Hsf2 promoter; murine COMP (cartilage oligomeric matrix protein) promoter; early B cell-specific mb-1 promoter 30 (Sigvardsson M., et al., 2002. Mol. Cell Biol. 22(24):8539-51); prostate specific antigen (PSA) promoter (Yoshimura I. et al., 2002, J. Urol. 168(6):2659-64); exorb promoter and pineal expression-promoting element (Asaoka Y., et al., 2002. Proc. Natl. Acad. Sci. 99(24):15456-61); neural and liver ceramidase gene promoters (Okino N. et al., 2002. Biochem. Biophys. Res. Commun. 299(1):160-6); PSP94 gene

promoter/enhancer (Gabril M.Y. et al., 2002. *Gene Ther.* 9(23):1589-99); promoter of the human FAT/CD36 gene (Kuriki C., et al., 2002. *Biol. Pharm. Bull.* 25(11):1476-8); VL30 promoter (Staplin W.R. et al., 2002. *Blood* October 24, 2002); and, IL-10 promoter (Brenner S., et al., 2002. *J. Biol. Chem.* December 18, 2002). Additional 5 promoters are shown in Table 1.

Examples of avian promoters include, but are not limited to, promoters controlling expression of egg white proteins, such as ovalbumin, ovotransferrin (conalbumin), ovomucoid, lysozyme, ovomucin, g2 ovoglobulin, g3 ovoglobulin, ovoflavoprotein, ovostatin (ovomacroglobin), cystatin, avidin, thiamine-binding 10 protein, glutamyl aminopeptidase minor glycoprotein 1, minor glycoprotein 2; and promoters controlling expression of egg-yolk proteins, such as vitellogenin, very low-density lipoproteins, low density lipoprotein, cobalamin-binding protein, riboflavin-binding protein, biotin-binding protein (Awade, 1996. *Z. Lebensm. Unters. Forsch.* 202:1-14). An advantage of using the vitellogenin promoter is that it is active during 15 the egg-laying stage of an animal's life-cycle, which allows for the production of the protein of interest to be temporally connected to the import of the protein of interest into the egg yolk when the protein of interest is equipped with an appropriate targeting sequence. In some embodiments, the avian promoter is an oviduct-specific promoter. As used herein, the term "oviduct-specific promoter" includes, but is not 20 limited to, ovalbumin; ovotransferrin (conalbumin); ovomucoid; 01, 02, 03, 04 or 05 avidin; ovomucin; g2 ovoglobulin; g3 ovoglobulin; ovoflavoprotein; and ovostatin (ovomacroglobin) promoters.

When germline transformation occurs via intraovarian or intratesticular administration, or when hepatocytes are targeted for incorporation of components of a 25 vector through non-germ line administration, liver-specific promoters may be operably-linked to the gene of interest to achieve liver-specific expression of the transgene. Liver-specific promoters of the present invention include, but are not limited to, the following promoters, vitellogenin promoter, G6P promoter, cholesterol-7-alpha-hydroxylase (CYP7A) promoter, phenylalanine hydroxylase 30 (PAH) promoter, protein C gene promoter, insulin-like growth factor I (IGF-I) promoter, bilirubin UDP-glucuronosyltransferase promoter, aldolase B promoter, furin promoter, metallothionein promoter, albumin promoter, and insulin promoter.

Also included in the present invention are promoters that can be used to target expression of a protein of interest into the milk of a milk-producing animal including,

but not limited to, β lactoglobulin promoter, whey acidic protein promoter, lactalbumin promoter and casein promoter.

When germline transformation occurs via intraovarian or intratesticular administration, or when cells of the immune system are targeted through non-germ line administration, immune system-specific promoters may be operably-linked to the gene of interest to achieve immune system-specific expression of the transgene. Accordingly, promoters associated with cells of the immune system may also be used. Acute phase promoters such as interleukin (IL)-1 and IL-2 may be employed. Promoters for heavy and light chain Ig may also be employed. The promoters of the T cell receptor components CD4 and CD8, B cell promoters and the promoters of CR2 (complement receptor type 2) may also be employed. Immune system promoters are preferably used when the desired protein is an antibody protein.

It is to be understood that any cell may be targeted for incorporation of a desired gene to provide gene therapy. Such cells may include, without limitation, endocrine cells or cancer cells. Promoters specific for selected endocrine cells, such as insulin-producing islet cells, hypophyseal growth hormone producing cells, or estrogen-producing follicular cells are known to one of ordinary skill in the art and may be incorporated into the transposon-based vectors of the present invention in order to provide gene therapy, by modulating hormone synthesis and secretion. Endocrine disorders and associated conditions of over or underproduction of hormones are known to one of ordinary skill in the art and many such conditions are described in textbooks such as Williams Textbook of Endocrinology, 10th ed., Williams, R.H. et al., eds. 2002, W.B. Saunders. Specific cancerous cells, such as ovarian cancer cells, are known to produce and release specific molecules. Promoters specific for these cells, and other cancerous cells, are known to one of ordinary skill in the art and may be incorporated into the transposon-based vectors of the present invention in order to provide gene therapy, perhaps by producing inhibitory RNA in these cells or by producing proteins or peptides that interfere with cancer cell function or replication.

Additional gene targets, especially related to cancer, are oncogenes and also genes involved in cellular functions such as cell division, microtubule production and spindle formation, growth factors, growth factor receptors, oncoproteins and signal transduction pathways associated with transducing signals associated with function of cancerous cells. It is to be understood that a gene target may be a particular protein or

enzyme involved in a multistep process associate with cell function. For example, an enzyme that is a component in a metabolic pathway of several enzymes may be disrupted using inhibitory RNA, thereby affecting the function of this metabolic pathway.

5 Also included in this invention are modified promoters/enhancers wherein elements of a single promoter are duplicated, modified, or otherwise changed. In one embodiment, steroid hormone-binding domains of the ovalbumin promoter are moved from about -3.5 kb to within approximately the first 1000 base pairs of the gene of interest. Modifying an existing promoter with promoter/enhancer elements not found
10 naturally in the promoter, as well as building an entirely synthetic promoter, or drawing promoter/enhancer elements from various genes together on a non-natural backbone, are all encompassed by the current invention.

Accordingly, it is to be understood that the promoters contained within the transposon-based vectors of the present invention may be entire promoter sequences
15 or fragments of promoter sequences. For example, in one embodiment, the promoter operably linked to a gene of interest is an approximately 900 base pair fragment of a chicken ovalbumin promoter (SEQ ID NO:21). The constitutive and inducible promoters contained within the transposon-based vectors may also be modified by the addition of one or more modified Kozak sequences of ACCATG (SEQ ID NO:8).

20 As indicated above, the present invention includes transposon-based vectors containing one or more enhancers. These enhancers may or may not be operably-linked to their native promoter and may be located at any distance from their operably-linked promoter. A promoter operably-linked to an enhancer and a promoter modified to eliminate repressive regulatory effects are referred to herein as an
25 "enhanced promoter." The enhancers contained within the transposon-based vectors may be enhancers found in birds, such as an ovalbumin enhancer, but are not limited to these types of enhancers. In one embodiment, an approximately 675 base pair enhancer element of an ovalbumin promoter is cloned upstream of an ovalbumin promoter with 300 base pairs of spacer DNA separating the enhancer and promoter.
30 In one embodiment, the enhancer used as a part of the present invention comprises base pairs 1-675 of a chicken ovalbumin enhancer from GenBank accession #S82527.1. The polynucleotide sequence of this enhancer is provided in SEQ ID NO:22.

Also included in some of the transposon-based vectors of the present invention are cap sites and fragments of cap sites. In one embodiment, approximately 50 base pairs of a 5' untranslated region wherein the capsite resides are added on the 3' end of an enhanced promoter or promoter. An exemplary 5' untranslated region is provided 5 in SEQ ID NO:23. A putative cap-site residing in this 5' untranslated region preferably comprises the polynucleotide sequence provided in SEQ ID NO:24.

In one embodiment of the present invention, the first promoter operably-linked to the transposase gene is a constitutive promoter and the second promoter operably-linked to the gene of interest is a cell specific promoter. In the second embodiment, 10 use of the first constitutive promoter allows for constitutive activation of the transposase gene and incorporation of the gene of interest into virtually all cell types, including the germline of the recipient animal. Although the gene of interest is incorporated into the germline generally, the gene of interest may only be expressed 15 in a tissue-specific manner to achieve gene therapy. A transposon-based vector having a constitutive promoter operably-linked to the transposase gene can be administered by any route, and in one embodiment, the vector is administered to an ovary, to an artery leading to the ovary or to a lymphatic system or fluid proximal to the ovary. In another embodiment, the transposon-based vector having a constitutive promoter operably-linked to the transposase gene can be administered to vessels 20 supplying the liver, muscle, brain, lung, kidney, heart or any other desired organ, tissue or cellular target.

It should be noted that cell- or tissue-specific expression as described herein does not require a complete absence of expression in cells or tissues other than the preferred cell or tissue. Instead, "cell-specific" or "tissue-specific" expression refers 25 to a majority of the expression of a particular gene of interest in the preferred cell or tissue, respectively.

When incorporation of the gene of interest into the germline is not preferred, the first promoter operably-linked to the transposase gene can be a tissue-specific promoter. For example, transfection of a transposon-based vector containing a 30 transposase gene operably-linked to a liver specific promoter such as the G6P promoter or vitellogenin promoter provides for activation of the transposase gene and incorporation of the gene of interest in the cells of the liver but not into the germline and other cells generally. In another example, transfection of a transposon-based vector containing a transposase gene operably-linked to an oviduct specific promoter

such as the ovalbumin promoter provides for activation of the transposase gene and incorporation of the gene of interest in the cells of the oviduct but not into the germline and other cells generally. In this embodiment, the second promoter operably-linked to the gene of interest can be a constitutive promoter or an inducible 5 promoter. In one embodiment, both the first promoter and the second promoter are an ovalbumin promoter. In embodiments wherein tissue-specific expression or incorporation is desired, it is preferred that the transposon-based vector is administered directly to the tissue of interest, to an artery leading to the organ or tissue of interest or to fluids surrounding the organ or tissue of interest. In one 10 embodiment, the tissue of interest is the oviduct and administration is achieved by direct injection into the oviduct or an artery leading to the oviduct. In another embodiment, the tissue of interest is the liver and administration is achieved by direct injection into the portal vein or hepatic artery. In another embodiment, the tissue of interest is cardiac muscle tissue in the heart and administration is achieved by direct 15 injection into the coronary arteries. In another embodiment, the tissue of interest is neural tissue and administration is achieved by direct injection into a cerebrovascular or spinovascular artery. In yet another embodiment, the target is a solid tumor and the administration is achieved by injection into a vessel supplying the tumor or by injection into the tumor. In yet another embodiment, the target is a diffuse cancer 20 such as ovarian cancer spread throughout the abdominopelvic cavity and the administration is achieved by injection into the abdominopelvic cavity. In yet another embodiment, the target is the lung, for example the surfactant-producing cells, and the administration is achieved by injection into the right ventricle, the pulmonary artery or a branch thereof, or by aerosol administration into the respiratory system. In still 25 another embodiment, the target is a lymph node and the administration is achieved by injection into lymphatic vessels supplying that node.

Accordingly, cell specific promoters may be used to enhance transcription in selected tissues. In birds, for example, promoters that are found in cells of the fallopian tube, such as ovalbumin, conalbumin, ovomucoid and/or lysozyme, are used 30 in the vectors to ensure transcription of the gene of interest in the epithelial cells and tubular gland cells of the fallopian tube, leading to synthesis of the desired protein encoded by the gene and deposition into the egg white. In mammals, promoters specific for the epithelial cells of the alveoli of the mammary gland, such as prolactin, insulin, beta lactoglobulin, whey acidic protein, lactalbumin, casein, and/or placental

lactogen, are used in the design of vectors used for transfection of these cells for the production of desired proteins for deposition into the milk. In liver cells, the G6P promoter may be employed to drive transcription of the gene of interest for protein production. Proteins made in the liver of birds may be delivered to the egg yolk.

5 In order to achieve higher or more efficient expression of the transposase gene, the promoter and other regulatory sequences operably-linked to the transposase gene may be those derived from the host. These host specific regulatory sequences can be tissue specific as described above or can be of a constitutive nature. For example, an avian actin promoter and its associated polyA sequence can be operably-linked to a transposase in a transposase-based vector for transfection into an avian. 10 Examples of other host specific promoters that could be operably-linked to the transposase include the myosin and DNA or RNA polymerase promoters.

Directing Sequences

15 In some embodiments of the present invention, the gene of interest is operably-linked to a directing sequence or a sequence that provides proper conformation to the desired protein encoded by the gene of interest. As used herein, the term "directing sequence" refers to both signal sequences and targeting sequences. An egg directing sequence includes, but is not limited to, an ovomucoid signal sequence, an ovalbumin signal sequence, a cecropin pre pro signal sequence, and a 20 vitellogenin targeting sequence. The term "signal sequence" refers to an amino acid sequence, or the polynucleotide sequence that encodes the amino acid sequence, that directs the protein to which it is linked to the endoplasmic reticulum in a eukaryote, and more preferably the translocational pores in the endoplasmic reticulum, or the 25 plasma membrane in a prokaryote, or mitochondria, such as for the purpose of gene therapy for mitochondrial diseases. Signal and targeting sequences can be used to direct a desired protein into, for example, the bloodstream, when the transposon-based vectors are administered to the liver of an animal.

Signal sequences can also be used to direct a desired protein into, for example, 30 a secretory pathway for secretion and release of the desired protein. For example appropriate signal sequences can be employed to provide gene therapy for enhanced secretion of growth hormone from selected cells, for example growth hormone cells, or liver cells. This therapy is useful for treating deficiencies in circulating growth

hormone levels and reduced stature. Liver specific promoters may be used to enhance production and release of antibodies or hepatic proteins such as globulins.

Signal sequences can also be used to direct a desired protein into, for example, a secretory pathway for incorporation into the egg yolk or the egg white, when the 5 transposon-based vectors are administered to a bird or other egg-laying animal. One example of such a transposon-based vector is provided in Figure 3 wherein the gene of interest is operably linked to the ovomucoid signal sequence. The present invention also includes a gene of interest operably-linked to a second gene containing a signal sequence. An example of such an embodiment is shown in Figure 2 wherein 10 the gene of interest is operably-linked to the ovalbumin gene that contains an ovalbumin signal sequence. Other signal sequences that can be included in the transposon-based vectors include, but are not limited to the ovotransferrin and lysozyme signal sequences. In one embodiment, the signal sequence is an ovalbumin signal sequence including a sequence shown in SEQ ID NO:25. In another 15 embodiment, the signal sequence is a modified ovalbumin signal sequence including a sequence shown in SEQ ID NO:26 or SEQ ID NO:27.

As also used herein, the term "targeting sequence" refers to an amino acid sequence, or the polynucleotide sequence encoding the amino acid sequence, which amino acid sequence is recognized by a receptor located on the exterior of a cell. 20 Binding of the receptor to the targeting sequence results in uptake of the protein or peptide operably-linked to the targeting sequence by the cell. One example of a targeting sequence is a vitellogenin targeting sequence that is recognized by a vitellogenin receptor (or the low density lipoprotein receptor) on the exterior of an oocyte. In one embodiment, the vitellogenin targeting sequence includes the 25 polynucleotide sequence of SEQ ID NO:28. In another embodiment, the vitellogenin targeting sequence includes all or part of the vitellogenin gene. Other targeting sequences include VLDL and Apo E, which are also capable of binding the vitellogenin receptor. Since the ApoE protein is not endogenously expressed in birds, its presence may be used advantageously to identify birds carrying the transposon-based vectors of the present invention. 30

Genes of Interest Encoding Desired Proteins

A gene of interest selected for stable incorporation is designed to encode any desired protein or peptide or nucleic acid or to regulate any cellular response. In some

embodiments, the desired proteins or peptides are released from cells into the surrounding environment, the lymphatic system or the vascular system. In some embodiments, the desired proteins or peptides are deposited in an egg or in milk. In other embodiments the desired proteins or peptides may be directed to an axon, to a 5 hepatocyte cell membrane for release into the bloodstream, to the membrane of a beta lymphocyte for release into the circulation. It is to be understood that the present invention encompasses transposon-based vectors containing multiple genes of interest. The multiple genes of interest may each be operably-linked to a separate promoter and other regulatory sequence(s) or may all be operably-linked to the same 10 promoter and other regulatory sequences(s). In one embodiment, multiple gene of interest are linked to a single promoter and other regulatory sequence(s) and each gene of interest is separated by a cleavage site or a pro portion of a signal sequence. A gene of interest may contain modifications of the codons for the first several N-terminal amino acids of the gene of interest, wherein the third base of each codon is 15 changed to an A or a T without changing the corresponding amino acid.

Protein and peptide hormones are a preferred class of proteins in the present invention. Such protein and peptide hormones are synthesized throughout the endocrine system and include, but are not limited to, hypothalamic hormones and hypophysiotropic hormones, anterior, intermediate and posterior pituitary hormones, 20 pancreatic islet hormones, hormones made in the gastrointestinal system, renal hormones, thymic hormones, parathyroid hormones, adrenal cortical and medullary hormones. Specifically, hormones that can be produced using the present invention include, but are not limited to, chorionic gonadotropin, corticotropin, erythropoietin, glucagons, IGF-1, oxytocin, platelet-derived growth factor, calcitonin, follicle- 25 stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, insulin, gonadotropin-releasing hormone and its analogs, vasopressin, octreotide, somatostatin, prolactin, adrenocorticotropic hormone, antidiuretic hormone, thyrotropin-releasing hormone (TRH), growth hormone-releasing hormone (GHRH), parathyroid hormone (PTH), glucagons, calcitrol, calciferol, atrial-natriuretic peptide, 30 gastrin, secretin, cholecystokinin (CCK), neuropeptide Y, ghrelin, PYY₃₋₃₆, angiotensinogen, thrombopoietin, and leptin. It is to be understood that proteins that are normally folded or have chains or component parts that combine to form the active protein may be produced in a linear manner, for example luteinizing hormone (LH).

Other multimeric proteins that may be produced using the present invention are as follows: factors involved in the synthesis or replication of DNA, such as DNA polymerase alpha and DNA polymerase delta; proteins involved in the production of mRNA, such as TFIID and TFIIH; cell, nuclear and other membrane-associated proteins, such as hormone and other signal transduction receptors, active transport proteins and ion channels, multimeric proteins in the blood, including hemoglobin, fibrinogen and von Willibrand's Factor; proteins that form structures within the cell, such as actin, myosin, and tubulin and other cytoskeletal proteins; proteins that form structures in the extra cellular environment, such as collagen, elastin and fibronectin; proteins involved in intra- and extra-cellular transport, such as kinesin and dynein, the SNARE family of proteins (soluble NSF attachment protein receptor) and clathrin; proteins that help regulate chromatin structure, such as histones and protamines, Swi3p, Rsc8p and moira; multimeric transcription factors such as Fos , Jun and CBTF (CCAAT box transcription factor); multimeric enzymes such as acetylcholinesterase and alcohol dehydrogenase; chaperone proteins such as GroE, Gro EL (chaperonin 60) and Gro ES (chaperonin 10); anti-toxins, such as snake venom, botulism toxin, Streptococcus super antigens; lysins (enzymes from bacteriophage and viruses); as well as most allosteric proteins. By using appropriate polynucleotide sequences, species-specific hormones may be made by transgenic animals.

In one embodiment of the present invention, the gene of interest is a proinsulin gene and the desired molecule is insulin. Proinsulin consists of three parts: a C-peptide and two strands of amino acids (the alpha and beta chains) that later become linked together to form the insulin molecule. Figures 2 and 3 are schematics of transposon-based vector constructs containing a proinsulin gene operably-linked to an ovalbumin promoter and ovalbumin protein or an ovomucoid promoter and ovomucoid signal sequence, respectively. In these embodiments, proinsulin is expressed in the oviduct tubular gland cells and then deposited in the egg white. One example of a proinsulin polynucleotide sequence is shown in SEQ ID NO:29, wherein the C-peptide cleavage site spans from Arg at position 31 to Arg at position 65. In other embodiments, the construct is designed for stable incorporation into hepatocytes and production of insulin for release into the vascular system.

In another embodiment of the present invention a vector is constructed for use in gene therapy for diabetes (Figure 6). A hepatocyte specific promoter is placed upstream of the transposase (ATS). The hepatocyte specific promoter could be a

glucose-6-phosphatase promoter, liver specific albumin promoter, serum alpha-fetoprotein promoter, or other hepatocyte specific promoter. Such a specific promoter permits gene incorporation into the genome of hepatocytes since the transposase would not be expressed in other cell types. The promoter driving expression of the 5 proinsulin gene is more specific, such as the glucose-6-phosphatase promoter. This promoter is desirable because it responds to blood glucose levels similar to beta islet cells of the pancreas. The proinsulin also contains a signal sequence to allow secretion from the liver cell. For instance, a signal sequence can be an albumin signal sequence or alpha-fetoprotein signal sequence. Likewise, the poly A is from a liver 10 specific protein in order to optimize mRNA stability for the amount of desired expression. This is easily determined by one skilled in the art. The proinsulin and liver specific sequences are from the species of animal targeted for gene therapy, i.e., human sequence for human gene therapy, canine sequence for canine gene therapy or feline sequences for gene therapy in felines.

15 In another embodiment of the present invention a vector is constructed for use in gene therapy for treatment of growth hormone deficiency by expressing growth hormone from hepatocytes (Figure 7). A liver specific promoter limits incorporation of the gene to hepatocytes. To further limit expression to hepatocytes, the vector is delivered as linear DNA as opposed to supercoiled DNA. Linear DNA has the added 20 advantage of being destroyed more quickly than supercoiled DNA, so that if the DNA were delivered to a cell and the promoter was leaky (a low basal level of expression), the chances of expression before degradation would be minimized. The selection of growth hormone expression level is related to the dosage desired, i.e. strong constitutive promoter for larger doses, low to intermediate constitutive promoter for 25 smaller doses. The signal sequence and poly A are hepatocyte derived for proper secretion and mRNA stability, respectively.

In another embodiment of the present invention a vector is constructed for use in gene therapy for treatment of cystic fibrosis by specifically expressing the normal CFTR gene in respiratory epithelial cells (Figure 8). To incorporate the desired 30 transgene into respiratory epithelial cells, the ciliated cell-specific promoter (FOXJ1), or another lung specific promoter, is used to drive expression of the transposase. To treat the disease, a normal CFTR gene is delivered to the respiratory epithelial cells .

In another embodiment of the present invention a vector is constructed for use in gene therapy for treatment of cancer by specifically expressing the cholera toxin

gene in cancer cells (Figure 9). By linking the transposase to a cancer specific promoter, only cancer cells are stably transformed with the target gene. The target gene can encode for a toxin, such as cholera toxin A, expressed constitutively or under control of a cancer specific promoter to selectively kill the transfected cancer cells. In another embodiment, the transposase is placed under control of a cell specific promoter so that only one cell is transformed. In one embodiment, the target gene is a secreted fusion peptide, such as a peptide that has a component recognized by a surface receptor on a cancer cell and a lytic component that would destroy the cell following the binding of the other part of the fusion peptide to the cell surface receptor. In one embodiment, the target gene could encode for betaLH/Phor14, which is a ligand/lytic peptide combination that targets a receptor on a cancer cell with LH receptors and kills that cell with little or no damage to surrounding healthy tissue.

Serum proteins including lipoproteins such as high density lipoprotein (HDL), HDL-Milano and low density lipoprotein, apolipoprotein, albumin, clotting cascade factors, factor VIII, factor IX, fibrinogen, and globulins are also included in the group of desired proteins of the present invention. Immunoglobulins are one class of desired globulin molecules and include but are not limited to IgG, IgM, IgA, IgD, IgE, IgY, lambda chains, kappa chains and fragments thereof; bi-specific antibodies, and fragments thereof; scFv fragments, Fc fragments, and Fab fragments as well as dimeric, trimeric and oligomeric forms of antibody fragments. Desired antibodies include, but are not limited to, naturally occurring antibodies, animal-specific antibodies, human antibodies, humanized antibodies, autoantibodies and hybrid antibodies. Genes encoding modified versions of naturally occurring antibodies or fragments thereof and genes encoding artificially designed antibodies or fragments thereof may be incorporated into the transposon-based vectors of the present invention. Desired antibodies also include antibodies with the ability to bind specific ligands, for example, antibodies against proteins associated with cancer-related molecules, such as anti-her 2, or anti-CA125. Accordingly, the present invention encompasses a transposon-based vector containing one or more genes encoding a heavy immunoglobulin (Ig) chain and a light Ig chain. Further, more than one gene encoding for more than one antibody may be administered in one or more transposon-based vectors of the present invention. In this manner, antibodies may be made in liver cells or another cell selected for transfection, such as fibroblasts and released locally or gain access to the circulation. In one embodiment, a transposon-based

vector contains a heavy Ig chain and a light Ig chain, both operably linked to a promoter.

Antibodies used as therapeutic reagents include but are not limited to antibodies for use in cancer immunotherapy against specific antigens, or for providing 5 passive immunity to an animal against an infectious disease or a toxic agent. Antibodies may be made by the animal receiving the transposon-based vectors to facilitate the animal's immune response to a selected antigen. Animals receiving gene therapy to enhance resistance to a disease or to fight an ongoing disease, such as cancer, may receive a transposon-based vector containing genes encoding antibodies 10 that bind to epitopes on cancer cells.

Antibodies that may be made with the practice of the present invention include, but are not limited to primary antibodies, secondary antibodies, designer antibodies, anti-protein antibodies, anti-peptide antibodies, anti-DNA antibodies, anti-RNA antibodies, anti-hormone antibodies, anti-hypophysiotropic peptides, antibodies 15 against non-natural antigens, anti-anterior pituitary hormone antibodies, anti-posterior pituitary hormone antibodies, anti-venom antibodies, anti-tumor marker antibodies, antibodies directed against epitopes associated with infectious disease, including, anti-viral, anti-bacterial, anti-protozoal, anti-fungal, anti-parasitic, anti-receptor, anti-lipid, anti-phospholipid, anti-growth factor, anti-cytokine, anti-monokine, anti-idiotype, and 20 anti-accessory (presentation) protein antibodies. Antibodies made with the present invention, as well as light chains or heavy chains, may also be used to inhibit enzyme activity.

Antibodies that may be produced using the present invention include, but are not limited to, antibodies made against the following proteins: Bovine γ -Globulin, 25 Serum; Bovine IgG, Plasma; Chicken γ -Globulin, Serum; Human γ -Globulin, Serum; Human IgA, Plasma; Human IgA₁, Myeloma; Human IgA₂, Myeloma; Human IgA₂, Plasma; Human IgD, Plasma; Human IgE, Myeloma; Human IgG, Plasma; Human IgG, Fab Fragment, Plasma; Human IgG, F(ab')₂ Fragment, Plasma; Human IgG, Fc Fragment, Plasma; Human IgG₁, Myeloma; Human IgG₂, Myeloma; Human IgG₃, 30 Myeloma; Human IgG₄, Myeloma; Human IgM, Myeloma; Human IgM, Plasma; Human Immunoglobulin, Light Chain κ , Urine; Human Immunoglobulin, Light Chains κ and λ , Plasma; Mouse γ -Globulin, Serum; Mouse IgG, Serum; Mouse IgM, Myeloma; Rabbit γ -Globulin, Serum; Rabbit IgG, Plasma; and Rat γ -Globulin, Serum. In one embodiment, the transposon-based vector comprises the coding

sequence of light and heavy chains of a murine monoclonal antibody that shows specificity for human seminoprotein (GenBank Accession numbers AY129006 and AY129304 for the light and heavy chains, respectively).

A further non-limiting list of antibodies that recognize other antibodies is as follows: Anti-Chicken IgG, heavy (H) & light (L) Chain Specific (Sheep); Anti-Goat γ -Globulin (Donkey); Anti-Goat IgG, Fc Fragment Specific (Rabbit); Anti-Guinea Pig γ -Globulin (Goat); Anti-Human Ig, Light Chain, Type κ Specific; Anti-Human Ig, Light Chain, Type λ Specific; Anti-Human IgA, α -Chain Specific (Goat); Anti-Human IgA, Fab Fragment Specific; Anti-Human IgA, Fc Fragment Specific; Anti-Human IgA, Secretory; Anti-Human IgE, ϵ -Chain Specific (Goat); Anti-Human IgE, Fc Fragment Specific; Anti-Human IgG, Fc Fragment Specific (Goat); Anti-Human IgG, γ -Chain Specific (Goat); Anti-Human IgG, Fc Fragment Specific; Anti-Human IgG, Fd Fragment Specific; Anti-Human IgG, H & L Chain Specific (Goat); Anti-Human IgG₁, Fc Fragment Specific; Anti-Human IgG₂, Fc Fragment Specific; Anti-Human IgG₂, Fd Fragment Specific; Anti-Human IgG₃, Hinge Specific; Anti-Human IgG₄, Fc Fragment Specific; Anti-Human IgM, Fc Fragment Specific; Anti-Human IgM, μ -Chain Specific; Anti-Mouse IgE, ϵ -Chain Specific; Anti-Mouse γ -Globulin (Goat); Anti-Mouse IgG, γ -Chain Specific (Goat); Anti-Mouse IgG, γ -Chain Specific (Goat) F(ab')₂ Fragment; Anti-Mouse IgG, H & L Chain Specific (Goat); Anti-Mouse IgM, μ -Chain Specific (Goat); Anti-Mouse IgM, H & L Chain Specific (Goat); Anti-Rabbit γ -Globulin (Goat); Anti-Rabbit IgG, Fc Fragment Specific (Goat); Anti-Rabbit IgG, H & L Chain Specific (Goat); Anti-Rat γ -Globulin (Goat); Anti-Rat IgG, H & L Chain Specific; Anti-Rhesus Monkey γ -Globulin (Goat); and, Anti-Sheep IgG, H & L Chain Specific.

Another non-limiting list of the antibodies that may be produced using the present invention is provided in product catalogs of companies such as Phoenix Pharmaceuticals, Inc. (www.phoenixpeptide.com; 530 Harbor Boulevard, Belmont, CA), Peninsula Labs (San Carlos CA), SIGMA (St. Louis, MO www.sigma-aldrich.com), Cappel ICN (Irvine, California, www.icnbiomed.com), and Calbiochem (La Jolla, California, www.calbiochem.com), which are all incorporated herein by reference in their entirety. The polynucleotide sequences encoding these antibodies may be obtained from the scientific literature, from patents, and from databases such as GenBank. Alternatively, one of ordinary skill in the art may design the polynucleotide sequence to be incorporated into the genome by choosing the codons

that encode for each amino acid in the desired antibody. Antibodies made by the transgenic animals of the present invention include antibodies that may be used as therapeutic reagents, for example in cancer immunotherapy against specific antigens. Some of these antibodies include, but are not limited to, antibodies which bind the following ligands: adrenomedulin, amylin, calcitonin, amyloid, calcitonin gene-related peptide, cholecystokinin, gastrin, gastric inhibitory peptide, gastrin releasing peptide, interleukin, interferon, cortistatin, somatostatin, endothelin, sarafotoxin, glucagon, glucagon-like peptide, insulin, atrial natriuretic peptide, BNP, CNP, neurokinin, substance P, leptin, neuropeptide Y, melanin concentrating hormone, melanocyte stimulating hormone, orphanin, endorphin, dynorphin, enkephalin, enkephalin, leumorphin, peptide F, PACAP, PACAP-related peptide, parathyroid hormone, urocortin, corticotrophin releasing hormone, PHM, PHI, vasoactive intestinal polypeptide, secretin, ACTH, angiotensin, angiotatin, bombesin, endostatin, bradykinin, FMRF amide, galanin, gonadotropin releasing hormone (GnRH) associated peptide, GnRH, growth hormone releasing hormone, inhibin, granulocyte-macrophage colony stimulating factor (GM-CSF), motilin, neurotensin, oxytocin, vasopressin, osteocalcin, pancreastatin, pancreatic polypeptide, peptide YY, proopiomelanocortin, transforming growth factor, vascular endothelial growth factor, vesicular monoamine transporter, vesicular acetylcholine transporter, ghrelin, NPW, NPB, C3d, prokinetican, thyroid stimulating hormone, luteinizing hormone, follicle stimulating hormone, prolactin, growth hormone, beta-lipotropin, melatonin, kallikriens, kinins, prostaglandins, erythropoietin, p146 (SEQ ID NO:30 amino acid sequence, SEQ ID NO:31, nucleotide sequence), estrogen, testosterone, corticosteroids, mineralocorticoids, thyroid hormone, thymic hormones, connective tissue proteins, nuclear proteins, actin, avidin, activin, agrin, albumin, and prohormones, propeptides, splice variants, fragments and analogs thereof.

The following is yet another non-limiting list of antibodies that can be produced by the methods of present invention: abciximab (ReoPro), abciximab anti-platelet aggregation monoclonal antibody, anti-CD11a (hu1124), anti-CD18 antibody, anti-CD20 antibody, anti-cytomegalovirus (CMV) antibody, anti-digoxin antibody, anti-hepatitis B antibody, anti-HER-2 antibody, anti-idiotype antibody to GD3 glycolipid, anti-IgE antibody, anti-IL-2R antibody, antimetastatic cancer antibody (mAb 17-1A), anti-rabies antibody, anti-respiratory syncytial virus (RSV) antibody, anti-Rh antibody, anti-TCR, anti-TNF antibody, anti-VEGF antibody and Fab

fragment thereof, rattlesnake venom antibody, black widow spider venom antibody, coral snake venom antibody, antibody against very late antigen-4 (VLA-4), C225 humanized antibody to EGF receptor, chimeric (human & mouse) antibody against TNF α , antibody directed against GPIIb/IIIa receptor on human platelets, gamma 5 globulin, anti-hepatitis B immunoglobulin, human anti-D immunoglobulin, human antibodies against *S. aureus*, human tetanus immunoglobulin, humanized antibody against the epidermal growth receptor-2, humanized antibody against the α subunit of the interleukin-2 receptor, humanized antibody CTLA4IG, humanized antibody to the IL-2 R α -chain, humanized anti-CD40-ligand monoclonal antibody (5c8), humanized 10 mAb against the epidermal growth receptor-2, humanized mAb to rous sarcoma virus, humanized recombinant antibody (IgG1k) against respiratory syncytial virus (RSV), lymphocyte immunoglobulin (anti-thymocyte antibody), lymphocyte immunoglobulin, mAb against factor VII, MDX-210 bi-specific antibody against HER-2, MDX-22, MDX-220 bi-specific antibody against TAG-72 on tumors, MDX- 15 33 antibody to Fc γ R1 receptor, MDX-447 bi-specific antibody against EGF receptor, MDX-447 bispecific humanized antibody to EGF receptor, MDX-RA immunotoxin (ricin A linked) antibody, Medi-507 antibody (humanized form of BTI-322) against CD2 receptor on T-cells, monoclonal antibody LDP-02, muromonab-CD3(OKT3) antibody, OKT3 ("muromomab-CD3") antibody, PRO 542 antibody, ReoPro 20 ("abciximab") antibody, and TNF-IgG fusion protein. It is to be understood that wherever the term "humanized" appears in the present patent application with regard to an antibody or molecule, that an antibody or molecule may be designed to be specific for any animal using selected polynucleotide sequences in the gene of interest included in the transposon-based vectors. Antibodies may be made against any 25 selected antigen known to one of ordinary skill in the art.

The antibodies prepared using the methods of the present invention may also be designed to possess specific labels that may be detected through means known to one of ordinary skill in the art so that their location and distribution can be assessed following gene therapy and expression of the antibodies. The antibodies may also be 30 designed to possess specific sequences useful for purification through means known to one of ordinary skill in the art. Specialty antibodies designed for binding specific antigens may also be made in transgenic animals using the transposon-based vectors of the present invention.

Production of a monoclonal antibody using the transposon-based vectors of the present invention can be accomplished in a variety of ways. In one embodiment, two vectors may be constructed: one that encodes the light chain, and a second vector that encodes the heavy chain of the monoclonal antibody. These vectors may then be 5 incorporated into the genome of the target animal by methods disclosed herein. In an alternative embodiment, the sequences encoding light and heavy chains of a monoclonal antibody may be included on a single DNA construct. For example, the coding sequence of light and heavy chains of a murine monoclonal antibody that show specificity for human seminoprotein can be expressed using transposon-based 10 constructs of the present invention (GenBank Accession numbers AY129006 and AY129304 for the light and heavy chains, respectively).

The transposon based vectors may include genes encoding proteins and peptides synthesized by the immune system including those synthesized by the thymus, lymph nodes, spleen, and the gastrointestinal associated lymph tissues 15 (GALT) system. The immune system proteins and peptides proteins that can be made in transgenic animals using the transposon-based vectors of the present invention include, but are not limited to, alpha-interferon, beta-interferon, gamma-interferon, alpha-interferon A, alpha-interferon 1, G-CSF, GM-CSF, interlukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TNF- α , and TNF- 20 β . Other cytokines included in the present invention include cardiotrophin, stromal cell derived factors including stromal cell derived factor alpha, macrophage derived chemokine (MDC), melanoma growth stimulatory activity (MGSA), macrophage inflammatory proteins 1 alpha (MIP-1 alpha), 2, 3 alpha, 3 beta, 4 and 5, heat shock proteins (HSP) of different molecular weights (HSP-70, HSP-80, HSP-90 and others). 25 Cell repellant molecules may also be made using the present invention, such as interleukins, stromal cell derived factor alpha and HSPs.

Lytic peptides, such as p146, are also included in the desired molecules that may be produced using the vectors and methods of the present invention. Lytic peptides are known to one of ordinary skill in the art and may be administered for 30 gene therapy, for example to lyse cancer cells. In one embodiment, the p146 peptide comprises an amino acid sequence of SEQ ID NO:30. The present invention also encompasses a transposon-based vector comprising a p146 nucleic acid comprising a polynucleotide sequence of SEQ ID NO:31. Other lytic peptides and the class of

proteins called lysins may be made with the transposon-based vectors of the present invention.

Enzymes are another class of proteins that may be made through gene therapy of the transposon-based vectors of the present invention. Such enzymes include but 5 are not limited to adenosine deaminase, alpha-galactosidase, cellulase, collagenase, dnaseI, hyaluronidase, lactase, L-asparaginase, pancreatin, papain, streptokinase B, subtilisin, superoxide dismutase, thrombin, trypsin, urokinase, fibrinolysin, 10 glucocerebrosidase and plasminogen activator. Many diseases, such as genetic diseases, involve problems in the production of enzymes. Through the practice of the 15 present invention, administration of the transposon based vectors encoding specific enzymes provides gene therapy to the animal or human. Examples of such conditions are known to one of ordinary skill in the art and include phenylketonuria, Tay-Sachs disease, and severe combined immunodeficiency disease, associated respectively with phenylalanine hydroxylase, hexosaminidase, and adenine deaminase. Other genetic 20 disorders are described in Robbins Pathologic Basis of Disease, Cotran et al. eds. 6th ed., pp 139-187, 1999 Saunders, and in Harrison's Principles of Internal Medicine, Fauci et al. eds. 14th ed. pp. 365-409, 1998, McGraw Hill. In some embodiments wherein the enzyme could have deleterious effects, additional amino acids and a protease cleavage site are added to the carboxy end of the enzyme of interest in order 25 to prevent expression of a functional enzyme. Subsequent digestion of the enzyme with a protease results in activation of the enzyme.

Extracellular matrix proteins are one class of desired proteins that may be made through the gene therapy methods of the present invention. Examples include but are not limited to collagen, fibrin, elastin, laminin, and fibronectin and subtypes 25 thereof. Animals receiving gene therapy for conditions such as arthritis or clotting disorders may make some of these matrix proteins. Gene therapy may be administered to stimulate formation of cartilage, such as articular cartilage, or for deposition of new bone. Intracellular proteins and structural proteins are other classes of desired proteins in the present invention.

30 Growth factors are another desired class of proteins that may be made through the gene therapy methods of the present invention and include, but are not limited to, transforming growth factor- α ("TGF- α "), transforming growth factor- β (TGF- β), platelet-derived growth factors (PDGF), fibroblast growth factors (FGF), including

FGF acidic isoforms 1 and 2, FGF basic form 2 and FGF 4, 8, 9 and 10, nerve growth factors (NGF) including NGF 2.5s, NGF 7.0s and beta NGF and neurotrophins, brain derived neurotrophic factor, cartilage derived factor, growth factors for stimulation of the production of red blood cells, growth factors for stimulation of the production of 5 white blood cells, bone growth factors (BGF), basic fibroblast growth factor, vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor (G-CSF), insulin like growth factor (IGF) I and II, hepatocyte growth factor, glial neurotrophic growth factor (GDNF), stem cell factor (SCF), keratinocyte growth factor (KGF), transforming growth factors (TGF), including TGFs alpha, beta, beta1, beta2, beta3, 10 skeletal growth factor, bone matrix derived growth factors, bone derived growth factors, erythropoietin (EPO) and mixtures thereof.

Another desired class of proteins that may be made may be made through the gene therapy of the present invention include, but are not limited to, leptin, leukemia inhibitory factor (LIF), tumor necrosis factor alpha and beta, ENBREL, angiostatin, 15 endostatin, thrombospondin, osteogenic protein-1, bone morphogenetic proteins 2 and 7, osteonectin, somatomedin-like peptide, and osteocalcin.

Yet another desired class of proteins are blood proteins or clotting cascade protein including albumin, Prekallikrein, High molecular weight kininogen (HMWK) (contact activation cofactor; Fitzgerald, Flaujeac Williams factor), Factor I 20 (Fibrinogen), Factor II (prothrombin), Factor III (Tissue Factor), Factor IV (calcium), Factor V (proaccelerin, labile factor, accelerator (Ac-) globulin), Factor VI (Va) (accelerin), Factor VII (proconvertin), serum prothrombin conversion accelerator (SPCA), cothromboplastin), Factor VIII (antihemophilic factor A, antihemophilic globulin (AHG)), Factor IX (Christmas Factor, antihemophilic factor B, plasma 25 thromboplastin component (PTC)), Factor X (Stuart-Prower Factor), Factor XI (Plasma thromboplastin antecedent (PTA)), Factor XII (Hageman Factor), Factor XIII (protransglutaminase, fibrin stabilizing factor (FSF), fibrinoligase), von Willibrand factor, Protein C, Protein S, Thrombomodulin, Antithrombin III.

A non-limiting list of the peptides and proteins that may be made may be 30 made through the use of the gene therapy methods of the present invention is provided in product catalogs of companies such as Phoenix Pharmaceuticals, Inc. (www.phoenixpeptide.com; 530 Harbor Boulevard, Belmont, CA), Peninsula Labs (San Carlos CA), SIGMA, (St. Louis, MO www.sigma-aldrich.com), Cappel ICN (Irvine, California, www.icnbiomed.com), and Calbiochem (La Jolla, California,

www.calbiochem.com). The polynucleotide sequences encoding these proteins and peptides of interest may be obtained from the scientific literature, from patents, and from databases, such as GenBank. Alternatively, one of ordinary skill in the art may design the polynucleotide sequence to be incorporated into the genome by choosing 5 the codons that encode for each amino acid in the desired protein or peptide.

Some of these desired proteins or peptides that may be made through the use of the gene therapy methods of the present invention include but are not limited to the following: adrenomedulin, amylin, calcitonin, amyloid, calcitonin gene-related peptide, cholecystokinin, gastrin, gastric inhibitory peptide, gastrin releasing peptide, 10 interleukin, interferon, cortistatin, somatostatin, endothelin, sarafotoxin, glucagon, glucagon-like peptide, insulin, atrial natriuretic peptide, BNP, CNP, neurokinin, substance P, leptin, neuropeptide Y, melanin concentrating hormone, melanocyte stimulating hormone, orphanin, endorphin, dynorphin, enkephalin, leumorphin, peptide F, PACAP, PACAP-related peptide, parathyroid hormone, urocortin, 15 corticotrophin releasing hormone, PHM, PHI, vasoactive intestinal polypeptide, secretin, ACTH, angiotensin, angiostatin, bombesin, endostatin, bradykinin, FMRF amide, galanin, gonadotropin releasing hormone (GnRH) associated peptide, GnRH, growth hormone releasing hormone, inhibin, granulocyte-macrophage colony stimulating factor (GM-CSF), motilin, neurotensin, oxytocin, vasopressin, 20 osteocalcin, pancreastatin, pancreatic polypeptide, peptide YY, proopiomelanocortin, transforming growth factor, vascular endothelial growth factor, vesicular monoamine transporter, vesicular acetylcholine transporter, ghrelin, NPW, NPB, C3d, prokineticin, thyroid stimulating hormone, luteinizing hormone, follicle stimulating hormone, prolactin, growth hormone, beta-lipotropin, melatonin, kallikriens, kinins, 25 prostaglandins, erythropoietin, p146 (SEQ ID NO:30, amino acid sequence, SEQ ID NO:31, nucleotide sequence), thymic hormones, connective tissue proteins, nuclear proteins, actin, avidin, activin, agrin, albumin, apolipoproteins, apolipoprotein A, apolipoprotein B, and prohormones, propeptides, splice variants, fragments and analogs thereof.

30 Other desired proteins that may be made by the transgenic animals receiving gene therapy according to the present invention include bacitracin, polymixin b, vancomycin, cyclosporine, anti-RSV antibody, alpha-1 antitrypsin (AAT), anti-cytomegalovirus antibody, anti-hepatitis antibody, anti-inhibitor coagulant complex, anti-rabies antibody, anti-Rh(D) antibody, adenosine deaminase, anti-digoxin

antibody, antivenin crotalidae (rattlesnake venom antibody), antivenin latroductus (black widow spider venom antibody), antivenin micrurus (coral snake venom antibody), aprotinin, corticotropin (ACTH), diphtheria antitoxin, lymphocyte immune globulin (anti-thymocyte antibody), protamine, thyrotropin, capreomycin, α -galactosidase, gramicidin, streptokinase, tetanus toxoid, tyrothricin, IGF-1, proteins of varicella vaccine, anti-TNF antibody, anti-IL-2r antibody, anti-HER-2 antibody, 5 OKT3 ("muromonab-CD3") antibody, TNF-IgG fusion protein, ReoPro ("abciximab") antibody, ACTH fragment 1-24, desmopressin, gonadotropin-releasing hormone, histrelin, leuprolide, lypressin, nafarelin, peptide that binds GPIIb/GPIIIa on 10 platelets (integrilin), goserelin, capreomycin, colistin, anti-respiratory syncytial virus, lymphocyte immune globulin (Thymoglovin, Atgam), panorex, alpha-antitrypsin, botulinin, lung surfactant protein, tumor necrosis receptor-IgG fusion protein (enbrel), gonadorelin, proteins of influenza vaccine, proteins of rotavirus vaccine, proteins of haemophilus b conjugate vaccine, proteins of poliovirus vaccine, proteins of 15 pneumococcal conjugate vaccine, proteins of meningococcal C vaccine, proteins of influenza vaccine, megakaryocyte growth and development factor (MGDF), neuroimmunophilin ligand-A (NIL-A), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), leptin (native), leptin B, leptin C, IL-1RA (interleukin-1RA), R-568, novel erythropoiesis-stimulating protein (ESP), 20 humanized mAb to rous sarcoma virus (MEDI-493), glutamyl-tryptophan dipeptide IM862, LFA-3TIP immunosuppressive, humanized anti-CD40-ligand monoclonal antibody (5c8), gelsonin enzyme, tissue factor pathway inhibitor (TFPI), proteins of meningitis B vaccine, antimetastatic cancer antibody (mAb 17-1A), chimeric (human & mouse) mAb against TNF α , mAb against factor VII, relaxin, capreomycin, 25 glycopeptide (LY333328), recombinant human activated protein C (rhAPC), humanized mAb against the epidermal growth receptor-2, alteplase, anti-CD20 antigen, C2B8 antibody, insulin-like growth factor-1, atrial natriuretic peptide (anaritide), tenectaplate, anti-CD11a antibody (hu 1124), anti-CD18 antibody, mAb LDP-02, anti-VEGF antibody, Fab fragment of anti-VEGF Ab, APO2 ligand (tumor 30 necrosis factor-related apoptosis-inducing ligand), rTGF- β (transforming growth factor- β), alpha-antitrypsin, ananain (a pineapple enzyme), humanized mAb CTLA4IG, PRO 542 (mAb), D2E7 (mAb), calf intestine alkaline phosphatase, α -L-iduronidase, α -L-galactosidase (human glutamic acid decarboxylase, acid

sphingomyelinase, bone morphogenetic protein-2 (rhBMP-2), proteins of HIV vaccine, T cell receptor (TCR) peptide vaccine, TCR peptides, V beta 3 and V beta 13.1. (IR502), (IR501), BI 1050/1272 mAb against very late antigen-4 (VLA-4), C225 humanized mAb to EGF receptor, anti-idiotype antibody to GD3 glycolipid, 5 antibacterial peptide against *H. pylori*, MDX-447 bispecific humanized mAb to EGF receptor, anti-cytomegalovirus (CMV), Medi-491 B19 parvovirus vaccine, humanized recombinant mAb (IgG1k) against respiratory syncytial virus (RSV), urinary tract infection vaccine (against "pili" on *Escherechia coli* strains), proteins of lyme disease vaccine against *B. burgdorferi* protein (DbpA), proteins of Medi-501 human 10 papilloma virus-11 vaccine (HPV), *Streptococcus pneumoniae* vaccine, Medi-507 mAb (humanized form of BTI-322) against CD2 receptor on T-cells, MDX-33 mAb to Fc_YR1 receptor, MDX-RA immunotoxin (ricin A linked) mAb, MDX-210 bi-specific mAb against HER-2, MDX-447 bi-specific mAb against EGF receptor, MDX-22, MDX-220 bi-specific mAb against TAG-72 on tumors, colony-stimulating 15 factor (CSF) (molgramostim), humanized mAb to the IL-2 R α -chain (basiliximab), mAb to IgE (IGE 025A), myelin basic protein-altered peptide (MSP771A), humanized mAb against the epidermal growth receptor-2, humanized mAb against the α subunit of the interleukin-2 receptor, low molecular weight heparin, anti-hemophilic factor, and bactericidal/permeability-increasing protein (r-BPI).

20 The peptides and proteins made by animals receiving gene therapy using the present invention may be labeled using labels and techniques known to one of ordinary skill in the art. Some of these labels are described in the "Handbook of Fluorescent Probes and Research Products", ninth edition, Richard P. Haugland (ed) Molecular Probes, Inc. Eugene, OR, which is incorporated herein in its entirety. 25 Some of these labels may be genetically engineered into the polynucleotide sequence for the expression of the selected protein or peptide. The peptides and proteins may also have label-incorporation "handles" incorporated to allow labeling of an otherwise difficult or impossible to label protein.

30 It is to be understood that the various classes of desired peptides and proteins, as well as specific peptides and proteins described in this section may be modified as described below by inserting selected codons for desired amino acid substitutions into the gene incorporated into the transgenic animal.

Genes of Interest Encoding Desired Nucleic Acids and Other Molecule

The present invention may also be used to produce desired molecules other than proteins and peptides including, but not limited to, lipoproteins such as high density lipoprotein (HDL), HDL-Milano, and low density lipoprotein, lipids, carbohydrates, siRNA and ribozymes. In these embodiments, a gene of interest 5 encodes a nucleic acid molecule or a protein that directs production of the desired molecule.

Nucleic Acids

RNAi technology can be directed against numerous aberrant genes, including those that allow proliferation of tumor cells. A variety of strategies can be used to 10 inhibit cancer. These include the inhibition of overexpressed oncogenes, blocking cell division by interfering with cyclin E and related genes or promoting apoptosis by suppressing antiapoptotic genes. RNAi against multidrug resistance genes or chemoresistance targets may also provide useful cancer treatments. A non-limiting list of gene and protein targets for cancer therapy is found in Table 2 (M. Izquierdo. 15 2004. Short interfering RNAs as a tool for cancer gene therapy. *Cancer Gene Therapy* pp 1-11).

There are guidelines for designing dsRNA for use as RNAi therapy. These rules are known to one of ordinary skill in the art. Generally, the dsRNA cannot be shorter than 21 nucleotides (nt) or longer than 30 nt so the antiviral interferon 20 response is not triggered. Other features to be avoided include, tight stem loops, inverted repeats, high sequence homology with other genes, and a lack of 4 or more consecutive T or A to avoid premature pol III transcription termination. Features to include are: 1) Initiation with a G or C after an AA in the 5' flanking sequence; 2) sense strand base preferences at positions 3 (A), 10 (U), 13 (A), and 19 (A); and 3) 25 low G/C content (30-60%) (M. Izquierdo. 2004. Short interfering RNAs as a tool for cancer gene therapy. *Cancer Gene Therapy* pp 1-11).

The present invention provides a new and effective method for delivering shRNA using transposon-based vectors. This method can be used to treat various conditions and diseases and is a method of providing gene therapy. shRNA would be 30 administered using a transposon based vector targeted to a specific cell type. The transposase (ATS) can be expressed by a cell-specific promoter (Table 1) to limit incorporation into a specific cell, and/or a cell-specific promoter could be used to express an shRNA to a gene listed in this document or any gene to be targeted for inactivation. In addition to the genes listed as targets for cancer therapy (see also

Table 3), genes such as apoB (apolipoprotein B) to lower cholesterol (Akinc, et al. 2004. *Nature* 432(7017): 155-156), viral genes to eliminate hepatitis B and C (Shlomai and Shaul. 2004. *Liver Int* 6:526-31), metabolism and obesity genes (Campion, et al. 2004 *Nutr. Rev* 62:321-330), HIV (Berkhout. 2004. *Curr Opin Mol Ther* 6:141-145; Takaku. 2004. *Antivir Chem Chemother*. 15:57-65), cardiac disease through down regulation of phospholamban (PL; Poller et al. 2004. *Z Kardiol* 93: 171-193), and 5' nontranslated region (5' NTR) of hepatitis C (Kronke et al. 2004. *J Virol*. 78:3436-3446).

The present invention further encompasses gene therapy to produce inhibitory molecules to inhibit endogenous (i.e., non-vector) protein production. Such therapy may be used to inhibit a gene that is over expressed. These inhibitory molecules include antisense nucleic acids, siRNA, polynucleotide strands that affect cellular function and inhibitory proteins. In one embodiment, the endogenous protein whose expression is inhibited is an egg white protein including, but not limited to ovalbumin, ovotransferrin, ovomucin, ovoinhibitor, cystatin, ovostatin, lysozyme, ovoglobulin G2, ovoglobulin G3, avidin, or thiamin binding protein. In one embodiment, a transposon-based vector containing an ovalbumin DNA sequence, that upon transcription forms a double stranded RNA molecule, is transfected into an animal, such as a bird, and the bird's production of endogenous ovalbumin protein is reduced by the interference RNA mechanism (RNAi). In other embodiments, a transposon-based vector encodes an inhibitory RNA molecule that inhibits the expression of more than one egg white protein. One exemplary construct is provided in Figure 4 wherein "Ovgen" indicates approximately 60 base pairs of an ovalbumin gene, "Ovotrans" indicates approximately 60 base pairs of an ovotransferrin gene and "Ovomucin" indicates approximately 60 base pairs of an ovomucin gene. These ovalbumin, ovotransferrin and ovomucin can be from any avian species, and in some embodiments, are from a chicken or quail. The term "pro" indicates the pro portion of a prepro sequence. One exemplary prepro sequence is that of cecropin and comprising base pairs 563-733 of the Cecropin cap site and Prepro provided in Genbank accession number X07404.

Additionally, inducible knockouts or knockdowns of the endogenous protein may be created to achieve a reduction or inhibition of endogenous protein production. The approach may be used for inhibition of any selected endogenous protein in animals receiving gene therapy.

Modified Desired Proteins and Peptides Made by Animals Receiving Gene Therapy

"Proteins", "peptides," "polypeptides" and "oligopeptides" are chains of amino acids (typically L-amino acids) whose alpha carbons are linked through peptide bonds 5 formed by a condensation reaction between the carboxyl group of the alpha carbon of one amino acid and the amino group of the alpha carbon of another amino acid. The terminal amino acid at one end of the chain (i.e., the amino terminal) has a free amino group, while the terminal amino acid at the other end of the chain (i.e., the carboxy terminal) has a free carboxyl group. As such, the term "amino terminus" (abbreviated 10 N-terminus) refers to the free alpha-amino group on the amino acid at the amino terminal of the protein, or to the alpha-amino group (imino group when participating in a peptide bond) of an amino acid at any other location within the protein. Similarly, the term "carboxy terminus" (abbreviated C-terminus) refers to the free carboxyl group on the amino acid at the carboxy terminus of a protein, or to the 15 carboxyl group of an amino acid at any other location within the protein.

Typically, the amino acids making up a protein are numbered in order, starting at the amino terminal and increasing in the direction toward the carboxy terminal of the protein. Thus, when one amino acid is said to "follow" another, that amino acid is positioned closer to the carboxy terminal of the protein than the preceding amino acid.

20 The term "residue" is used herein to refer to an amino acid (D or L) or an amino acid mimetic that is incorporated into a protein by an amide bond. As such, the amino acid may be a naturally occurring amino acid or, unless otherwise limited, may encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (i.e., amino acid mimetics). Moreover, an amide 25 bond mimetic includes peptide backbone modifications well known to those skilled in the art.

Furthermore, one of skill will recognize that, as mentioned above, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than about 5%, more typically less 30 than about 1%) in an encoded sequence are conservatively modified variations where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Such substitutions may be engineered by selecting the desired nucleotides for insertion into the gene of interest in animals receiving gene therapy. Conservative substitutions in polynucleotide sequences are included within the scope

of the present invention, wherein codons in a sequence may be replaced with other codons encoding for conservatively substituted amino acids, as explained below in the conservative substitution table. In other words, a codon in a polynucleotide sequence encoding for an alanine may be substituted with a codon encoding for a valine.

5 Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

10 1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
10 3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

15 A conservative substitution is a substitution in which the substituting amino acid (naturally occurring or modified) is structurally related to the amino acid being substituted, i.e., has about the same size and electronic properties as the amino acid being substituted. Thus, the substituting amino acid would have the same or a similar functional group in the side chain as the original amino acid. A "conservative substitution" also refers to utilizing a substituting amino acid which is identical to the 20 amino acid being substituted except that a functional group in the side chain is protected with a suitable protecting group.

25 Suitable protecting groups are described in Green and Wuts, "Protecting Groups in Organic Synthesis", John Wiley and Sons, Chapters 5 and 7, 1991, the teachings of which are incorporated herein by reference. Preferred protecting groups are those which facilitate transport of the peptide through membranes, for example, by reducing the hydrophilicity and increasing the lipophilicity of the peptide, and which can be cleaved, either by hydrolysis or enzymatically (Ditter et al., 1968. J. Pharm. Sci. 57:783; Ditter et al., 1968. J. Pharm. Sci. 57:828; Ditter et al., 1969. J. Pharm. Sci. 58:557; King et al., 1987. Biochemistry 26:2294; Lindberg et al., 1989. Drug 30 Metabolism and Disposition 17:311; Tunek et al., 1988. Biochem. Pharm. 37:3867; Anderson et al., 1985 Arch. Biochem. Biophys. 239:538; and Singhal et al., 1987. FASEB J. 1:220). Suitable hydroxyl protecting groups include ester, carbonate and carbamate protecting groups. Suitable amine protecting groups include acyl groups and alkoxy or aryloxy carbonyl groups, as described above for N-terminal protecting

groups. Suitable carboxylic acid protecting groups include aliphatic, benzyl and aryl esters, as described below for C-terminal protecting groups. In one embodiment, the carboxylic acid group in the side chain of one or more glutamic acid or aspartic acid residues in a peptide of the present invention is protected, preferably as a methyl, 5 ethyl, benzyl or substituted benzyl ester, more preferably as a benzyl ester.

Provided below are groups of naturally occurring and modified amino acids in which each amino acid in a group has similar electronic and steric properties. Thus, a conservative substitution can be made by substituting an amino acid with another amino acid from the same group. Such substitutions may be engineered through 10 selection of the appropriate nucleotides in constructing the gene of interest for introduction into animals receiving gene therapy. It is to be understood that these groups are non-limiting, i.e. that there are additional modified amino acids which could be included in each group.

Group I includes leucine, isoleucine, valine, methionine and modified amino acids 15 having the following side chains: ethyl, n-propyl n-butyl. Preferably, Group I includes leucine, isoleucine, valine and methionine.

Group II includes glycine, alanine, valine and a modified amino acid having an ethyl side chain. Preferably, Group II includes glycine and alanine.

Group III includes phenylalanine, phenylglycine, tyrosine, tryptophan, 20 cyclohexylmethyl glycine, and modified amino residues having substituted benzyl or phenyl side chains. Preferred substituents include one or more of the following: halogen, methyl, ethyl, nitro, —NH₂, methoxy, ethoxy and —CN. Preferably, Group III includes phenylalanine, tyrosine and tryptophan.

Group IV includes glutamic acid, aspartic acid, a substituted or unsubstituted 25 aliphatic, aromatic or benzylic ester of glutamic or aspartic acid (e.g., methyl, ethyl, n-propyl iso-propyl, cyclohexyl, benzyl or substituted benzyl), glutamine, asparagine, —CO—NH— alkylated glutamine or asparagines (e.g., methyl, ethyl, n-propyl and iso-propyl) and modified amino acids having the side chain —(CH₂)₃—COOH, an ester thereof (substituted or unsubstituted 30 aliphatic, aromatic or benzylic ester), an amide thereof and a substituted or unsubstituted N-alkylated amide thereof. Preferably, Group IV includes glutamic acid, aspartic acid, methyl aspartate, ethyl aspartate, benzyl aspartate and methyl glutamate, ethyl glutamate and benzyl glutamate, glutamine and asparagine.

Group V includes histidine, lysine, ornithine, arginine, N-nitroarginine, β -cycloarginine, γ -hydroxyarginine, N-amidinocitruline and 2-amino-4-guanidinobutanoic acid, homologs of lysine, homologs of arginine and homologs of ornithine. Preferably, Group V includes histidine, lysine, arginine and ornithine. A homolog of an amino acid includes from 1 to about 5 3 additional or subtracted methylene units in the side chain.

Group VI includes serine, threonine, cysteine and modified amino acids having C1-C5 straight or branched alkyl side chains substituted with —OH or —SH, for example, —CH₂CH₂OH, —CH₂CH₂CH₂OH or -CH₂CH₂OHCH₃. Preferably, 10 Group VI includes serine, cysteine or threonine.

In another aspect, suitable substitutions for amino acid residues include "severe" substitutions. A "severe substitution" is a substitution in which the substituting amino acid (naturally occurring or modified) has significantly different size and/or electronic properties compared with the amino acid being substituted. 15 Thus, the side chain of the substituting amino acid can be significantly larger (or smaller) than the side chain of the amino acid being substituted and/or can have functional groups with significantly different electronic properties than the amino acid being substituted. Examples of severe substitutions of this type include the substitution of phenylalanine or cyclohexylmethyl glycine for alanine, isoleucine for 20 glycine, a D amino acid for the corresponding L amino acid, or —NH—CH[(-CH₂)₅—COOH]—CO— for aspartic acid. Alternatively, a functional group may be added to the side chain, deleted from the side chain or exchanged with another functional group. Examples of severe substitutions of this type include adding of valine, leucine or isoleucine, exchanging the carboxylic acid in the side chain of 25 aspartic acid or glutamic acid with an amine, or deleting the amine group in the side chain of lysine or ornithine. In yet another alternative, the side chain of the substituting amino acid can have significantly different steric and electronic properties that the functional group of the amino acid being substituted. Examples of such modifications include tryptophan for glycine, lysine for aspartic acid and —(CH₂)₄COOH for the side chain of serine. These examples are not meant to be 30 limiting.

In another embodiment, for example in the synthesis of a peptide 26 amino acids in length, the individual amino acids may be substituted according in the following manner:

AA₁ is serine, glycine, alanine, cysteine or threonine;
AA₂ is alanine, threonine, glycine, cysteine or serine;
AA₃ is valine, arginine, leucine, isoleucine, methionine, ornithine, lysine, N-nitroarginine, β -cycloarginine, γ -hydroxyarginine, N-amidinocitruline or 2-amino-4-
5 guanidinobutanoic acid;
AA₄ is proline, leucine, valine, isoleucine or methionine;
AA₅ is tryptophan, alanine, phenylalanine, tyrosine or glycine;
AA₆ is serine, glycine, alanine, cysteine or threonine;
AA₇ is proline, leucine, valine, isoleucine or methionine;
10 AA₈ is alanine, threonine, glycine, cysteine or serine;
AA₉ is alanine, threonine, glycine, cysteine or serine;
AA₁₀ is leucine, isoleucine, methionine or valine;
AA₁₁ is serine, glycine, alanine, cysteine or threonine;
AA₁₂ is leucine, isoleucine, methionine or valine;
15 AA₁₃ is leucine, isoleucine, methionine or valine;
AA₁₄ is glutamine, glutamic acid, aspartic acid, asparagine, or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;
AA₁₅ is arginine, N-nitroarginine, β -cycloarginine, γ -hydroxy-arginine, N-amidinocitruline or 2-amino-4-guanidino-butanoic acid
20 AA₁₆ is proline, leucine, valine, isoleucine or methionine;
AA₁₇ is serine, glycine, alanine, cysteine or threonine;
AA₁₈ is glutamic acid, aspartic acid, asparagine, glutamine or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;
AA₁₉ is aspartic acid, asparagine, glutamic acid, glutamine, leucine, valine, isoleucine,
25 methionine or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;
AA₂₀ is valine, arginine, leucine, isoleucine, methionine, ornithine, lysine, N-nitroarginine, β -cycloarginine, γ -hydroxyarginine, N-amidinocitruline or 2-amino-4-
guanidinobutanoic acid;
30 AA₂₁ is alanine, threonine, glycine, cysteine or serine;
AA₂₂ is alanine, threonine, glycine, cysteine or serine;
AA₂₃ is histidine, serine, threonine, cysteine, lysine or ornithine;
AA₂₄ is threonine, aspartic acid, serine, glutamic acid or a substituted or unsubstituted
aliphatic or aryl ester of glutamic acid or aspartic acid;

AA₂₅ is asparagine, aspartic acid, glutamic acid, glutamine, leucine, valine, isoleucine, methionine or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid; and

AA₂₆ is cysteine, histidine, serine, threonine, lysine or ornithine.

5 It is to be understood that these amino acid substitutions may be made for longer or shorter peptides than the 26 mer in the preceding example above, and for proteins.

In one embodiment of the present invention, codons for the first several N-terminal amino acids of the transposase are modified such that the third base of each 10 codon is changed to an A or a T without changing the corresponding amino acid. It is preferable that between approximately 1 and 20, more preferably 3 and 15, and most preferably between 4 and 12 of the first N-terminal codons of the gene of interest are modified such that the third base of each codon is changed to an A or a T without changing the corresponding amino acid. In one embodiment, the first ten N-terminal 15 codons of the gene of interest are modified in this manner.

When several desired proteins, protein fragments or peptides are encoded in the gene of interest to be incorporated into the genome, one of skill in the art will appreciate that the proteins, protein fragments or peptides may be separated by a spacer molecule such as, for example, a peptide, consisting of one or more amino 20 acids. Generally, the spacer will have no specific biological activity other than to join the desired proteins, protein fragments or peptides together, or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of the spacer may be selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity. The spacer may also 25 be contained within a nucleotide sequence with a purification handle or be flanked by cleavage sites, such as proteolytic cleavage sites.

Such polypeptide spacers may have from about 1 to about 100 amino acids, preferably 3 to 20 amino acids, and more preferably 4-15 amino acids. The spacers in a polypeptide are independently chosen, but are preferably all the same. The 30 spacers should allow for flexibility of movement in space and are therefore typically rich in small amino acids, for example, glycine, serine, proline or alanine. Preferably, peptide spacers contain at least 60%, more preferably at least 80% glycine or alanine. In addition, peptide spacers generally have little or no biological and antigenic activity. Preferred spacers are (Gly-Pro-Gly-Gly)_x (SEQ ID NO:32) and (Gly₄-Ser)_y,

wherein x is an integer from about 3 to about 9 and y is an integer from about 1 to about 8. Specific examples of suitable spacers include

(Gly-Pro-Gly-Gly)₃

SEQ ID NO:33 Gly Pro Gly Gly Pro Gly Gly Pro Gly Gly

5 (Gly₄-Ser)₃

SEQ ID NO:34 Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser

or (Gly₄-Ser)₄

SEQ ID NO:35 Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser.

10 Nucleotide sequences encoding for the production of residues which may be useful in purification of the expressed recombinant protein in animals receiving gene therapy may also be built into the vector. Such sequences are known in the art and include the glutathione binding domain from glutathione S-transferase, polylysine, hexa-histidine or other cationic amino acids, thioredoxin, hemagglutinin antigen and 15 maltose binding protein.

Additionally, nucleotide sequences may be inserted into the gene of interest to be incorporated so that the protein or peptide can also include from one to about six amino acids that create signals for proteolytic cleavage. In this manner, if a gene is designed to make one or more peptides or proteins of interest in the transgenic animal, 20 specific nucleotide sequences encoding for amino acids recognized by enzymes may be incorporated into the gene to facilitate cleavage of the large protein or peptide sequence into desired peptides or proteins or both. For example, nucleotides encoding a proteolytic cleavage site can be introduced into the gene of interest so that a signal sequence can be cleaved from a protein or peptide encoded by the gene of interest. 25 Nucleotide sequences encoding other amino acid sequences which display pH sensitivity or chemical sensitivity may also be added to the vector to facilitate separation of the signal sequence from the peptide or protein of interest.

30 Proteolytic cleavage sites include cleavage sites recognized by exopeptidases such as carboxypeptidase A, carboxypeptidase B, aminopeptidase I, and dipeptidylaminopeptidase; endopeptidases such as trypsin, V8-protease, enterokinase, factor Xa, collagenase, endoproteinase, subtilisin, and thrombin; and proteases such as Protease 3C IgA protease (Igase) Rhinovirus 3C(preScission)protease. Chemical cleavage sites are also included in the definition of cleavage site as used herein.

Chemical cleavage sites include, but are not limited to, site cleaved by cyanogen bromide, hydroxylamine, formic acid, and acetic acid.

In one embodiment of the present invention, a TAG sequence is linked to the gene of interest. The TAG sequence serves three purposes: 1) it allows free rotation 5 of the peptide or protein to be isolated so there is no interference from the native protein or signal sequence, i.e. vitellogenin, 2) it provides a "purification handle" to isolate the protein using column purification, and 3) it includes a cleavage site to remove the desired protein from the signal and purification sequences. Accordingly, as used herein, a TAG sequence includes a spacer sequence, a purification handle and 10 a cleavage site. The spacer sequences in the TAG proteins contain one or more repeats shown in SEQ ID NO:36. A preferred spacer sequence comprises the sequence provided in SEQ ID NO:37. One example of a purification handle is the gp41 hairpin loop from HIV I. Exemplary gp41 polynucleotide and polypeptide sequences are provided in SEQ ID NO:38 and SEQ ID NO:39, respectively. 15 However, it should be understood that any antigenic region may be used as a purification handle, including any antigenic region of gp41. Preferred purification handles are those that elicit highly specific antibodies. Additionally, the cleavage site can be any protein cleavage site known to one of ordinary skill in the art and includes an enterokinase cleavage site comprising the Asp Asp Asp Asp Lys sequence (SEQ 20 ID NO:40) and a furin cleavage site. Constructs containing a TAG sequence are shown in Figures 2 and 3. In one embodiment of the present invention, the TAG sequence comprises a polynucleotide sequence of SEQ ID NO:41.

Gene Therapy

25 Administration of the transposon based vectors of the present invention to achieve gene therapy in animals may be used to treat numerous genetic and non-genetic disorders.

DNA constructs of the present invention can be used to transform any animal 30 cell, including but not limited to: cells producing hormones, cytokines, growth factors, or any other biologically active substance; cells of the immune system; cells of the nervous system; muscle (striatal, cardiac, smooth) cells; vascular system cells; endothelial cells; skin cells; mammary cells; and lung cells, including bronchial and alveolar cells. Transformation of any endocrine cell by a transposon-based DNA construct is contemplated as a part of a present invention. DNA constructs of the

present invention can be used to modulate, including both stimulation and inhibition, production of any substance, including but not limited to a hormone, a cytokine, or a growth factor, by an animal cell. Modulation of a regulated signal within a cell or a tissue, such as production of a second messenger, is also contemplated as a part of the 5 present invention. In one aspect of the present invention, cells of the immune system may be the target for incorporation of a desired gene or genes encoding for production of antibodies. Accordingly, the thymus, bone marrow, beta lymphocytes (or B cells), gastrointestinal associated lymphatic tissue (GALT), Peyer's patches, bursa Fabricius, lymph nodes, spleen, and tonsil, and any other lymphatic tissue, may all be targets for 10 administration of the compositions of the present invention. Use of the DNA constructs of the present invention is contemplated for treatment of any animal disease or condition that results from underproduction (such as diabetes) or overproduction (such as hyperthyroidism) of a hormone or other endogenous biologically active substance. Use of DNA constructs of the present invention to 15 integrate nucleotide sequences encoding RNA molecules, such as anti-sense RNA or short interfering RNA, is also contemplated as a part of the present invention.

Genetic disorders

Genetic disorders are well known to one of ordinary skill in the art and may 20 include, but are not limited to, general classes of mutations, Mendelian disorders, disorders with multifactorial inheritance, cytogenetic disorders, and single gene disorders with nonclassic inheritance. Many genetic disorders are described in Robbins Pathologic Basis of Disease, Cotran et al. eds. 6th ed., pp 139-187, 1999 Saunders, and in Harrison's Principles of Internal Medicine, Fauci et al. eds. 14th ed. 25 pp. 365-409, 1998, McGraw Hill. Genetic disorders that may be treated with the method of the present invention include, but are not limited to those presented in Table 3, which also identifies the gene and often the chromosome associated with the specific genetic disorder.

Mendelian disorders include autosomal dominant disorders, autosomal 30 recessive disorders and X-linked disorders. Such disorders may include defective enzymes, defects in receptor and transport systems, alterations in the structure, function or quality of non-enzyme proteins, and genetically determined adverse reactions to drugs. Some of these conditions are related to familial hypercholesterolemia, lysosomal storage diseases, glycogen storage diseases and

neurofibromatosis. Provision of gene therapy using the method of the present invention may address, for example, supplementation of an animal with a protein or enzyme that the animal needs in view of its inadequate or faulty production of the protein. Practice of the present invention can be used to inactivate the defective gene 5 through use of siRNA and then the transposon based vector can be used to insert the normal gene in order to restore function.

Other disorders

The present invention also provides gene therapy for animals that may not possess a demonstrable genetic deficiency. However, such animals may require 10 supplementation of specific proteins that may be produced in inadequate amounts or in a defective form that renders them biologically inactive or marginally active. Alternatively, animals may produce too much of a protein that causes a disease or condition that renders the animal sick. Such animals may require gene therapy to reduce the transcription of a gene that makes the protein. Such animals may require 15 gene therapy to produce proteins or peptides to blunt or block the activity of the overabundant protein.

Diseases and Conditions

Numerous diseases and conditions may be treated with the gene therapy 20 method of the present invention, including, but not limited to, diseases and conditions of the following systems: cardiovascular system (atherosclerosis, hypercholesterolemia, disorders of LDL, HDL and apolipoprotein synthesis and metabolism, hypertension); reproductive system (reproductive health and dysfunction, fertility, infertility, menopause, menarche, puberty, superovulation, timing of 25 ovulation, inducement of ovulation, inducement of sterilization (especially of companion animals), mastitis, cancers of the reproductive system); endocrine and neuroendocrine systems (hypopituitary disorders, hypothalamic disorders, hypogonadism, precocious puberty, dwarfism, infertility, lactation, diabetes, thyroid disease, adrenal cortical or adrenal medullary disease, appetite, feeding, drinking, 30 temperature regulation); metabolic system (digestive disorders, inborn errors of metabolism, disorders of intermediate metabolism, fat metabolism, Crohn's disease; phenylketonuria, chronic wasting disease, phosphofructokinase deficiency, pyruvic kinase deficiency; nervous system (Parkinson's disease, Alzheimer's disease, Huntington's disease, encephalopathy, bovine spongiform encephalopathy, conditions

related to neurotransmitter transporter systems such as catecholamine transporters and reuptake mechanisms (serotonin, norepinephrine, dopamine) such as depression, psychosis, neurosis, addiction, alcoholism, motivation, bulimia, hyperphagia); immune system (feline immunodeficiency virus, simian immunodeficiency virus, 5 immunodeficiency disorders including severe immunodeficiency disorders and severe combined immunodeficiency disorders, leukemia, autoimmune disorders, allergies, lupus, multiple sclerosis, scleroderma, disorders involving various immunoglobulins, interleukins, cytokines and lymphokines); hematologic and related disorders (sickle cell anemia, clotting disorders, von Willibrand's Disease); musculoskeletal system 10 (arthritis, rheumatoid arthritis, osteoarthritis, muscular dystrophy); cancer (ovarian, prostate, breast, colon, brain, lung, kidney, skin); respiratory system (lung cancer, laryngeal cancer, cystic fibrosis); obesity; aging; cosmetic treatment of skin and hair; any form of cancer (skin (melanoma, basal, squamous), bladder, colon, stomach, 15 esophageal, liver, pancreatic, testicular, prostate, ovarian, cervical, uterine, breast, lung, laryngeal, thyroid, adrenal, renal, penile, head, neck, brain (neural, glial); disorders involving receptors, particularly membrane bound receptors; and, infectious diseases (parasitic disease, bacterial infectious disease, viral disease, pneumovirus, Eastern equine encephalitis, West Nile virus, malaria, lyme disease, ehrlichiosis, retroviral infections, rabies, and diseases borne by invertebrates such as ticks, fleas, 20 flies and mosquitoes.

The transposon-based vectors of the present invention can be used for the treatment of various genetic disorders. For example, one or more LTR-vector complexes can be administered to an animal for the treatment of a single gene disorder including, but not limited to, animal equivalents of Huntington's disease, 25 alpha-1-antitrypsin deficiency Alzheimer's disease, various forms of breast cancer, cystic fibrosis, galactosemia, congenital hypothyroidism, maple syrup urine disease, neurofibromatosis 1, phenylketonuria, sickle cell disease, and Smith-Lemli-Opitz (SLO/RSH) Syndrome any metabolic errors, autoimmune diseases, shipping fever in cattle, mastitis, bacterial or viral diseases, alteration of skin pigment in animals, 30 production of animals with enhanced growth characteristics and nutrient utilization. In these embodiments, the transposon-based vector contains a non-mutated, or non-disease causing form of the gene known to cause such disorder. The transposon-based vectors of the present invention can also be used to treat multiple gene disorders. The transposon-based vectors of the present invention can be used as DNA

vaccines and are useful in organ-specific disease treatments and localized disease treatments.

Preferably, the transposase contained within the transposase-based vector is operably linked to an inducible promoter such as a tissue specific promoter such that 5 the non-mutated gene of interest is inserted into a specific tissue wherein the mutated gene is expressed *in vivo*. Additionally, the DNA constructs of the present invention can be used to provide cells or tissues with “beacons”, such as receptor molecules, for binding of therapeutic agents in order to provide tissue and cell specificity for the therapeutic agents. Several promoters and exogenous genes can be combined in one 10 vector to produce progressive, controlled, treatments, from a single vector delivery.

In avians, for example, one or more LTR-vector complexes are administered to an avian for the treatment of a viral or bacterial infection/disease including, but not limited to, *Colibacillosis* (Coliform infections), *Mycoplasmosis* (CRD, Air sac, Sinusitis), *Fowl Cholera*, *Necrotic Enteritis*, *Ulcerative Enteritis* (Quail disease), 15 *Pullorum Disease*, *Fowl Typhoid*, *Botulism*, *Infectious Coryza*, *Erysipelas*, *Avian Pox*, *Newcastle Disease*, *Infectious Bronchitis*, *Quail Bronchitis*, *Lymphoid Leukosis*, *Marek's Disease* (Visceral Leukosis), *Infectious Bursal Disease* (Gumboro), *Avian Encephalomyelitis* (AE), *Avian Influenza* (AI), *Avian Leukosis Virus* (LLAg, LLAb, ALV-J), *Reticuloendotheliosis Virus* (REV), *Avian Pneumovirus* (APV), *Chicken 20 Anemia Virus* (CAV), *Infectious Bronchitis Virus* (IBV), *Infectious Bursal Disease Virus* – Gumboro Disease (IBD, IBD-XR), *Mycoplasma* (MG, MS, MG/MS, MM), *Newcastle Disease Virus* (NDV, NDV-T), *Ornithobacterium rhinotracheale* (ORT), *Pasteurella multocida* (PM, PM-T), *Reovirus* (REO), and *Salmonella enteritidis* (SE).

In swine, for example, one or more transposon-based vectors are administered 25 for the treatment of a viral or bacterial infection/disease including, but not limited to, *Pseudorabies Virus* – Aujeszky's Disease (PRV-V, PRV-S, PRV gl (gE)), *Porcine Reproductive and Respiratory Syndrome* (PRRS 2XR), *Classical Swine Fever Virus* (CSFV Ab, CSFV Ag), *Swine Influenza* (SIV H1N1), *Mycoplasma hyopneumoniae* (M. hyo.), and *Swine Salmonella*.

30 In ruminants, for example, one or more transposon-based vectors are administered for the treatment of a viral or bacterial infection/disease including, but not limited to, *Bovine Leukemia Virus* (BLV), *Infectious Bovine Rhinotracheitis* (IBR, IBR gB, IBR gE), *Brucella abortus* (B. abortus), *Mycobacterium*

paratuberculosis – Johne's Disease (M. pt.), Neospora caninum, and Bovine Viral Diarrhea Virus (BVDV).

In horses, for example, one or more transposon-based vectors are administered for the treatment of a viral or bacterial infection/disease including, but not limited to,
5 Equine Infectious Anemia (EIA).

Numerous genetic diseases that affect humans are shown in Table 3.

Methods of Administering Compositions Comprising Transposon-Based Vectors to Provide Therapy

10 The compositions of the present invention, comprising a vector, a transfecting reagent and an acceptable carrier may be delivered to a desired location in an animal receiving gene therapy through administration via a selected route. Accordingly, the compositions may be administered in a variety of ways including, but not limited to the following: through a vascular system, a duct system, within the lumen of an organ, 15 into an organ, tissue or cell, into a body cavity, into the cerebrospinal fluid, topically, through the gastrointestinal system, through the reproductive system, through the urinary system, intraperitoneally, and through the respiratory system.

The vector can be administered into the vascular system. In a preferred embodiment, the vector is administered into the cardiovascular system and 20 specifically into one or more chambers of the heart. Administration of the vector into the cardiovascular system and specifically into one or more chambers of the heart, results in the distribution of the vector to the organs and tissues and cells receiving blood supply from the vessel or the heart. In a preferred embodiment, administration of the vector into the left ventricle of the heart results in distribution of the vector to 25 the organs supplied by branches of the aorta, for example the celiac, gonadal, superior (cranial) mesenteric and inferior (caudal) mesenteric arteries. Such distribution targets include the liver, ovary, oviduct and testes, among other organs. Administration through the internal mammary artery transfects secretory cells of the lactating mammary gland to perform a desired function, such as to synthesize and 30 secrete a desired protein or peptide into the milk. Administration through the internal mammary artery would also target breast cancer cells. Administration of the compositions into the artery supplying the ovary or to the fallopian tube to supply those tissues. In this manner, follicles are transfected to create a germline transgenic animal. Alternatively, supplying the compositions through the artery leading to the

5 fallopian tube preferably transfects the epithelial cells. Such transfected epithelial cells manufacture a desired protein or peptide for deposition in the egg white. Administration of the compositions through the portal vein or hepatic artery targets uptake and transformation of hepatic cells. Intravascular administration further includes administration into any vein, including but not limited to veins in the systemic circulation and veins in the hepatic portal circulation. Intravascular administration further includes administration into the cerebrovascular system, including the carotid arteries, the vertebral arteries and branches thereof.

10 Intravascular administration may be coupled with methods known to influence the permeability of vascular barriers such as the blood brain barrier and the blood testes barrier, in order to enhance transfection of cells that are difficult to affect through vascular administration. Such methods are known to one of ordinary skill in the art and include use of hyperosmotic agents, mannitol, hypothermia, nitric oxide, alkylglycerols, lipopolysaccharides (Haluska et al., Clin. J. Oncol. Nursing 8(3): 263-267, 2004; Brown et al., Brain Res., 1014: 221-227, 2004; Ikeda et al., Acta Neurochir. Suppl. 86:559-563, 2004; Weyerbrock et al., J. Neurosurg. 99(4):728-737, 2003; Erdlenbruch et al., Br. J. Pharmacol. 139(4):685-694, 2003; Gaillard et al., Microvasc. Res. 65(1):24-31, 2003; Lee et al., Biol. Reprod. 70(2):267-276, 2004)).

15 Intravascular administration may also be coupled with methods known to influence vascular diameter, such as use of beta blockers, nitric oxide generators, prostaglandins and other reagents that increase vascular diameter and blood flow.

20 In one embodiment, the animal is an egg-laying animal, and more preferably, an avian, and the transposon-based vectors comprising the polynucleotide cassettes are administered into the vascular system, preferably into the heart. In one embodiment, between approximately 1 and 300 μ g, 1 and 200 μ g, 5 and 200 μ g, or 5 and 150 μ g of a transposon-based vector containing the polynucleotide cassette is administered to the vascular system, preferably into the heart. In a chicken, it is preferred that between approximately 1 and 300 μ g, or 5 and 200 μ g are administered to the vascular system, preferably into the heart, more preferably into the left ventricle. The total injection volume for administration into the left ventricle of a chicken may range from about 10 μ l to about 3.0 ml, or from about 100 μ l to about 1.5 ml, or from about 200 μ l to about 1.0 ml, or from about 200 μ l to about 800 μ l. It is to be understood that the total injection volume may vary depending on the duration

of the injection. Longer injection durations may accommodate higher total volumes. In a quail, it is preferred that between approximately 1 and 200 μ g, or 5 and 150 μ g are administered to the vascular system, preferably into the heart, more preferably into the left ventricle. The total injection volume for administration into the left ventricle 5 of a quail may range from about 10 μ l to about 1.0 ml, or from about 100 μ l to about 800 μ l, or from about 200 μ l to about 600 μ l. It is to be understood that the total injection volume may vary depending on the duration of the injection. Longer injection durations may accommodate higher total volumes. The microgram quantities represent the total amount of the vector with the transfection reagent.

10 Other, non-avian animals will require different volumes and amounts for injection and these values can be extrapolated on a body weight or surface area basis as known to one of ordinary skill in the art. For example, an intravascular administration into a rat may occur through a cannula inserted into the right or left atrium or ventricle and may comprise a volume of from about 0.05 ml to 4 ml 15 containing 1 and 300 μ g is injected gradually.

Administration may also occur through non vascular routes. For example, administration through the urethra and into the bladder targets the transitional epithelium of the bladder. Administration through the vagina and cervix targets the lining of the uterus. For example, administration may occur directly into a muscle to 20 transfect striated muscle cells for production of a desired protein.

In one embodiment of the present invention, a transposon-based vector comprising a gene encoding proinsulin is administered to diabetic animals receiving gene therapy for incorporation into liver cells in order to treat or cure diabetes. The specific incorporation of the proinsulin gene into the liver is accomplished by placing 25 the transposase of the transposon-based vector under control of liver-specific promoter, such as the glucose-6-phosphatase promoter (G6P). This approach is useful for treatment of both type I and type II diabetes. The G6P promoter has been shown to be glucose responsive (Arguad, D., et al. 1996, *Diabetes* 45: 1563-1571), and thus, glucose-regulated insulin production is achieved using DNA constructs of the present 30 invention. Integrating a proinsulin gene into liver cells circumvents the problem of destruction of pancreatic islet cells in the course of type 1 diabetes.

In another embodiment, shortly after diagnosis of type I diabetes, the cells of the immune system destroying β -cells of the pancreas are selectively removed using

the DNA constructs of the present invention, thus allowing normal β -cells to repopulate the pancreas.

For treatment of type II diabetes, the DNA constructs of the present invention are specifically incorporated into the pancreas by placing the transposase of the 5 transposon-based vector under the control of a pancreas-specific promoter, such as an insulin promoter. In this embodiment, the vector is delivered to a diabetic animal via injection into an artery supplying the pancreas. For delivery, the vector is complexed with a transfection agent. The artery distributes the complex throughout the pancreas, where individual cells receive the vector DNA. Following uptake into the target cell, 10 the insulin promoter is recognized by transcriptional machinery of the cell, the transposase encoded by the vector is expressed, and stable integration of the proinsulin gene occurs. It is expected that a small percentage of the DNA construct would be transported to other tissues, and that these tissues would be transfected. However, these tissues would not be stably transfected due to failure of these other 15 cells to activate the insulin promoter. The DNA would likely be lost when the cell dies or degraded over time.

In addition to the transposon-based vectors described above, the present invention also includes methods of administering the transposon-based vectors to an animal, methods of producing a transgenic animal wherein a gene of interest is 20 incorporated into the germline of the animal and methods of producing a transgenic animal wherein a gene of interest is incorporated into cells other than the germline cells (somatic cells) of the animal. For example, the transposon-based vectors of the present invention are administered to a reproductive organ of an animal via any method known to those of skill in the art. Preferred reproductive organs include a 25 testis, an ovary, an oviduct, a mammary gland, and a fallopian tube.

In some embodiments, a transposon-based vector is directly administered to the reproductive organ. Direct administration encompasses injection into the organ, and in one embodiment, a transposon-based vector is injected into the lumen of the oviduct, and more preferably, the lumen of the magnum or the infundibulum of the 30 oviduct. The transposon-based vectors may additionally or alternatively be placed in an artery supplying the reproductive organ. Administering the vectors to the artery supplying the ovary results in transfection of follicles and oocytes in the ovary to create a germline transgenic animal. Alternatively, supplying the vectors through an

artery leading to the oviduct would preferably transfect the tubular gland and epithelial cells. Such transfected cells manufacture a desired protein or peptide for deposition in the egg white. In one embodiment, a transposon-based vector is administered into the lumen of the magnum or the infundibulum of the oviduct and to 5 an artery supplying the oviduct. Indirect administration to the oviduct epithelium may occur through the cloaca. Direct administration into the mammary gland may be achieved through introduction into the duct system of the mammary gland or an artery supplying the mammary gland.

The transposon-based vectors may be administered in a single administration, 10 multiple administrations, continuously, or intermittently. The transposon-based vectors may be administered by injection, via a catheter, an osmotic mini-pump or any other method. In some embodiments, the transposon-based vector is administered to an animal in multiple administrations, each administration containing the vector and a different transfecting reagent.

15 The transposon-based vectors may be administered to the animal at any desirable time for gene therapy during the lifetime of the animal.

In one embodiment, between approximately 1 μ g and 5 mg, 1 μ g and 3 mg, 1 μ g and 1 mg, of transposon-based vector DNA is administered to the animal. Intraoviduct administration of the transposon-based vectors of the present invention 20 resulted in incorporation of the gene of interest into the cells of the oviduct as evidenced by a PCR positive signal in the oviduct tissue, demonstrating that the present invention is effective in providing genetic therapy to the animal. In other embodiments, the transposon-based vector is administered to an artery that supplies the oviduct. These methods of administration may also be combined with any 25 methods for facilitating transfection, including without limitation, electroporation, gene guns, injection of naked DNA, and use of dimethyl sulfoxide (DMSO).

According to the present invention, the transposon-based vector is administered in conjunction with an acceptable carrier and/or transfection reagent. Acceptable carriers include, but are not limited to, water, saline, Hanks Balanced Salt 30 Solution (HBSS), Tris-EDTA (TE) and lyotropic liquid crystals. Transfection reagents commonly known to one of ordinary skill in the art that may be employed include, but are not limited to, the following: cationic lipid transfection reagents, cationic lipid mixtures, polyamine reagents, liposomes and combinations thereof;

SUPERFECT®, Cytofectene, BioPORTER®, GenePORTER®, NeuroPORTER®, and perfectin from Gene Therapy Systems; lipofectamine, cellfectin, DMRIE-C oligofectamine, and PLUS reagent from InVitrogen; Xtreme gene, fugene, DOSPER and DOTAP from Roche; Lipotaxi and Genejammer from Strategene; and Escort 5 from SIGMA. In one embodiment, the transfection reagent is SUPERFECT®. The ratio of DNA to transfection reagent may vary based upon the method of administration. In one embodiment, the transposon-based vector is administered to the oviduct and the ratio of DNA to transfection reagent can be from 1:1.5 to 1:15, preferably 1:2 to 1:5, all expressed as wt/vol. Transfection may also be accomplished 10 using other means known to one of ordinary skill in the art, including without limitation electroporation, gene guns, injection of naked DNA, and use of dimethyl sulfoxide (DMSO).

Depending upon the cell or tissue type targeted for transfection, the form of the transposon-based vector may be important. Plasmids harvested from bacteria are 15 generally closed circular supercoiled molecules, and this is the preferred state of a vector for gene delivery because of the ease of preparation. In some instances, transposase expression and insertion may be more efficient in a relaxed, closed circular configuration or in a linear configuration. In still other instances, a purified transposase protein may be co-injected with a transposon-based vector containing the 20 gene of interest for more immediate insertion. This could be accomplished by using a transfection reagent complexed with both the purified transposase protein and the transposon-based vector.

Testing for and Breeding Animals Carrying the Transgene

Following administration of a transposon-based vector to an animal receiving 25 gene therapy, DNA is extracted from the animal to confirm integration of the gene of interest. Advantages provided by the present invention include the high rates of integration, or incorporation, and transcription of the gene of interest when administered to a bird via an intraoviduct or intraovarian route (including intraarterial 30 administrations to arteries leading to the oviduct or ovary). The construct of Figure 2, when administered to Japanese quail hens, resulted in expression of the fusion peptide in the oviduct cells and subsequent secretion and deposition in the egg white. Assaying of the egg white on and SDS PAGE gel demonstrated the presence of the expressed protein. The sequence of the fusion protein was verified by MALDI-TOF

analysis by an independent third party. The proinsulin/ENT TAG protein from a transgenic hen was isolated following ammonium sulfate precipitation and ion exchange chromatography. The transposon-based vector was successfully administered to a hen, and the gene of interest successfully integrated. The protein 5 encoded by the gene of interest was produced and deposited in egg white produced by the transgenic hen.

Actual frequencies of integration may be estimated both by comparative strength of the PCR signal, and by histological evaluation of the tissues by quantitative PCR. Another method for estimating the rate of transgene insertion is the 10 so-called primed *in situ* hybridization technique (PRINS). This method determines not only which cells carry a transgene of interest, but also into which chromosome the gene has inserted, and even what portion of the chromosome. Briefly, labeled primers are annealed to chromosome spreads (affixed to glass slides) through one round of PCR, and the slides are then developed through normal *in situ* hybridization 15 procedures. This technique combines the best features of *in situ* PCR and fluorescence *in situ* hybridization (FISH) to provide distinct chromosome location and copy number of the gene in question. The 28s rRNA gene will be used as a positive control for spermatogonia to confirm that the technique is functioning properly. Using different fluorescent labels for the transgene and the 28s gene causes cells 20 containing a transgene to fluoresce with two different colored tags.

Breeding experiments are also conducted to determine if germline transmission of the transgene has occurred. In a general bird breeding experiment performed according to the present invention, each male bird was exposed to 2-3 different adult female birds for 3-4 days each. This procedure was continued with 25 different females for a total period of 6-12 weeks. Eggs are collected daily for up to 14 days after the last exposure to the transgenic male, and each egg is incubated in a standard incubator. The resulting embryos are examined for transgene presence at day 3 or 4 using PCR. It is to be understood that the above procedure can be modified to suit animals other than birds and that selective breeding techniques may be 30 performed to amplify gene copy numbers and protein output.

Production of Desired Proteins or Peptides in Egg White

In one embodiment, the transposon-based vectors of the present invention may be administered to a bird receiving gene therapy for production of desired proteins or

peptides in the egg white. These transposon-based vectors preferably contain one or more of an ovalbumin promoter, an ovomucoid promoter, an ovalbumin signal sequence and an ovomucoid signal sequence. Oviduct-specific ovalbumin promoters are described in B. O'Malley et al., 1987. *EMBO J.*, vol. 6, pp. 2305-12; A. Qiu et al., 5 1994. *Proc. Nat. Acad. Sci. (USA)*, vol. 91, pp. 4451-4455; D. Monroe et al., 2000. *Biochim. Biophys. Acta*, 1517 (1):27-32; H. Park et al., 2000. *Biochem.*, 39:8537-8545; and T. Muramatsu et al., 1996. *Poult. Avian Biol. Rev.*, 6:107-123. Examples of transposon-based vectors designed for production of a desired protein in an egg white are shown in Figures 2 and 3.

10

Production of Desired Proteins or Peptides in Egg Yolk

The present invention is particularly advantageous for production of recombinant peptides and proteins of low solubility in the egg yolk. Such proteins include, but are not limited to, membrane-associated or membrane-bound proteins, 15 lipophilic compounds; attachment factors, receptors, and components of second messenger transduction machinery. Low solubility peptides and proteins are particularly challenging to produce using conventional recombinant protein production techniques (cell and tissue cultures) because they aggregate in water-based, hydrophilic environments. Such aggregation necessitates denaturation and re-folding of the recombinantly-produced proteins, which may deleteriously affect their 20 structure and function. Moreover, even highly soluble recombinant peptides and proteins may precipitate and require denaturation and renaturation when produced in sufficiently high amounts in recombinant protein production systems. The present invention provides an advantageous resolution of the problem of protein and peptide 25 solubility during production of large amounts of recombinant proteins.

In one embodiment of the present invention wherein germline transfection is obtained via intraovarian administration of the transposon-based vector, deposition of a desired protein into the egg yolk is accomplished in offspring by attaching a sequence encoding a protein capable of binding to the yolk vitellogenin receptor to a 30 gene of interest that encodes a desired protein. This transposon-based vector can be used for the receptor-mediated uptake of the desired protein by the oocytes. In a preferred embodiment, the sequence ensuring the binding to the vitellogenin receptor is a targeting sequence of a vitellogenin protein. The invention encompasses various vitellogenin proteins and their targeting sequences. In a preferred embodiment, a

chicken vitellogenin protein targeting sequence is used, however, due to the high degree of conservation among vitellogenin protein sequences and known cross-species reactivity of vitellogenin targeting sequences with their egg-yolk receptors, other vitellogenin targeting sequences can be substituted. One example of a construct 5 for use in the transposon-based vectors of the present invention and for deposition of an insulin protein in an egg yolk is a transposon-based vector containing a vitellogenin promoter, a vitellogenin targeting sequence, a TAG sequence, a pro-insulin sequence and a synthetic polyA sequence. The present invention includes, but is not limited to, vitellogenin targeting sequences residing in the N-terminal domain 10 of vitellogenin, particularly in lipovitellin I. In one embodiment, the vitellogenin targeting sequence contains the polynucleotide sequence of SEQ ID NO:28. In a preferred embodiment, the transposon-based vector contains a transposase gene operably-linked to a constitutive promoter and a gene of interest operably-linked to a liver-specific promoter and a vitellogenin targeting sequence.

15 The following examples will serve to further illustrate the present invention without, at the same time, however, constituting any limitation thereof. On the contrary, it is to be clearly understood that resort may be had to various embodiments, modifications and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the 20 invention.

EXAMPLE 1

Preparation of Transposon-Based Vector pTnMod

A vector was designed for inserting a desired coding sequence into the 25 genome of eukaryotic cells, given below as SEQ ID NO:7. The vector of SEQ ID NO:7, termed pTnMod, was constructed and its sequence verified.

This vector employed a cytomegalovirus (CMV) promoter. A modified Kozak sequence (ACCATG) (SEQ ID NO:8) was added to the promoter. The nucleotide in the wobble position in nucleotide triplet codons encoding the first 10 amino acids of 30 transposase was changed to an adenine (A) or thymine (T), which did not alter the amino acid encoded by this codon. Two stop codons were added and a synthetic polyA was used to provide a strong termination sequence. This vector uses a promoter designed to be active soon after entering the cell (without any induction) to increase the likelihood of stable integration. The additional stop codons and synthetic

polyA insures proper termination without read through to potential genes downstream.

The first step in constructing this vector was to modify the transposase to have the desired changes. Modifications to the transposase were accomplished with the 5 primers High Efficiency forward primer (Hef) Altered transposase (ATS)-Hef 5' ATCTCGAGACCATGTGTGAACTTGATATTACATGAATTCTCTTACC 3' (SEQ ID NO:42) and Altered transposase- High efficiency reverse primer (Her) 5' GATTGATCATTATCATAATTCCCCAAAGCGTAACC 3' (SEQ ID NO:43, a reverse complement primer). The sequence ACCATG (SEQ ID NO:8) contains the 10 Kozak sequence and start codon for the transposase and the underlined bases represent changes in the wobble position to an A or T of codons for the first 10 amino acids (without changing the amino acid coded by the codon). Primer ATS-Her (SEQ ID NO:43) contains an additional stop codon TAA in addition to native stop codon TGA and adds a Bcl I restriction site to allow directional cloning. These primers were 15 used in a PCR reaction with pTnLac (p defines plasmid, tn defines transposon, and lac defines the beta fragment of the lactose gene, which contains a multiple cloning site) as the template for the transposase and a FailSafe™ PCR System (which includes enzyme, buffers, dNTP's, MgCl₂ and PCR Enhancer; Epicentre Technologies, Madison, WI). Amplified PCR product was electrophoresed on a 1% agarose gel, 20 stained with ethidium bromide, and visualized on an ultraviolet transilluminator. A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA). Purified DNA was digested with restriction enzymes Xho I (5') and Bcl I (3') (New 25 England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from restriction enzymes using a Zymo DNA Clean and Concentrator kit (Zymo Research).

Plasmid gWhiz (Gene Therapy Systems, San Diego, CA) was digested with restriction enzymes Sal I and BamH I (New England Biolabs), which are compatible with Xho I and Bcl I, but destroy the restriction sites. Digested gWhiz was separated 30 on an agarose gel, the desired band excised and purified as described above. Cutting the vector in this manner facilitated directional cloning of the modified transposase (mATS) between the CMV promoter and synthetic polyA.

To insert the mATS between the CMV promoter and synthetic polyA in gWhiz, a Stratagene T4 Ligase Kit (Stratagene, Inc. La Jolla, CA) was used and the

ligation set up according to the manufacturer's protocol. Ligated product was transformed into *E. coli* Top10 competent cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to Invitrogen's protocol. Transformed bacteria were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) medium for 1 hour at 37° C before being spread to LB (Luria-Bertani media (broth or agar)) plates supplemented with 100 µg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37° C and resulting colonies picked to LB/amp broth for overnight growth at 37° C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on a U.V. transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size (approximately 6.4 kbp) were cultured in at least 250 ml of LB/amp broth and plasmid DNA harvested using a Qiagen Maxi-Prep Kit (column purification) according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). Column purified DNA was used as template for sequencing to verify the changes made in the transposase were the desired changes and no further changes or mutations occurred due to PCR amplification. For sequencing, Perkin-Elmer's Big Dye Sequencing Kit was used. All samples were sent to the Gene Probes and Expression Laboratory (LSU School of Veterinary Medicine) for sequencing on a Perkin-Elmer Model 377 Automated Sequencer.

Once a clone was identified that contained the desired mATS in the correct orientation, primers CMVf-NgoM IV and Syn-polyA-BstE II were used to PCR amplify the entire CMV promoter, mATS, and synthetic polyA for cloning upstream of the transposon in pTnLac. The PCR was conducted with FailSafe™ as described above, purified using the Zymo Clean and Concentrator kit, the ends digested with NgoM IV and BstE II (New England Biolabs), purified with the Zymo kit again and cloned upstream of the transposon in pTnLac as described below.

Plasmid pTnLac was digested with NgoM IV and BstE II to remove the ptac promoter and transposase and the fragments separated on an agarose gel. The band corresponding to the vector and transposon was excised, purified from the agarose, and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs) to prevent self-annealing. The enzyme was removed from the vector using a Zymo DNA Clean and Concentrator-5. The purified vector and CMVp/mATS/polyA

were ligated together using a Stratagene T4 Ligase Kit and transformed into *E. coli* as described above.

Colonies resulting from this transformation were screened (mini-preps) as describe above and clones that were the correct size were verified by DNA sequence 5 analysis as described above. The vector was given the name pTnMod (SEQ ID NO:7) and includes the following components:

Base pairs 1-130 are a remainder of F1(-) on from pBluescriptII sk(-) (Stratagene), corresponding to base pairs 1-130 of pBluescriptII sk(-).

10 Base pairs 131 - 132 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 133 -1777 are the CMV promoter/enhancer taken from vector pGWiz (Gene Therapy Systems), corresponding to bp 229-1873 of pGWiz. The CMV promoter was modified by the addition of an ACC sequence upstream of ATG.

15 Base pairs 1778-1779 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 1780 - 2987 are the coding sequence for the transposase, modified from Tn10 (GenBank accession J01829) by optimizing codons for stability of the transposase mRNA and for the expression of protein. More specifically, in each of the codons for the first ten amino acids of the transposase, G or C was changed to A or T 20 when such a substitution would not alter the amino acid that was encoded.

Base pairs 2988-2993 are two engineered stop codons.

Base pair 2994 is a residue from ligation of restriction enzyme sites used in constructing the vector.

25 Base pairs 2995 - 3410 are a synthetic polyA sequence taken from the pGWiz vector (Gene Therapy Systems), corresponding to bp 1922-2337 of 10 pGWiz.

Base pairs 3415 - 3718 are non-coding DNA that is residual from vector pNK2859.

Base pairs 3719 - 3761 are non-coding λ DNA that is residual from pNK2859.

30 Base pairs 3762 - 3831 are the 70 bp of the left insertion sequence recognized by the transposon Tn10.

Base pairs 3832-3837 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 3838 - 4527 are the multiple cloning site from pBluescriptII sk(20), corresponding to bp 924-235 of pBluescriptII sk(-). This multiple cloning site may be used to insert any coding sequence of interest into the vector.

5 Base pairs 4528-4532 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 4533 - 4602 are the 70 bp of the right insertion sequence recognized by the transposon Tn10.

Base pairs 4603 - 4644 are non-coding λ DNA that is residual from pNK2859.

10 Base pairs 4645 - 5488 are non-coding DNA that is residual from pNK2859.

10 Base pairs 5489 - 7689 are from the pBluescriptII sk(-) base vector - (Stratagene, Inc.), corresponding to bp 761-2961 of pBluescriptII sk(-).

Completing pTnMod is a pBlueScript backbone that contains a *colE* I origin of replication and an antibiotic resistance marker (ampicillin).

15 It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences in the above construct represent restriction site remnants.

20 All plasmid DNA was isolated by standard procedures. Briefly, *Escherichia coli* containing the plasmid was grown in 500 mL aliquots of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight with shaking. Plasmid DNA was recovered from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 μ L of PCR-grade water and stored at -20°C until used.

EXAMPLE 2

25 *Transposon-Based Vector pTnMCS*

Another transposon-based vector was designed for inserting a desired coding sequence into the genome of eukaryotic cells. This vector was termed pTnMCS and its constituents are provided below. The sequence of the pTnMCS vector is provided in SEQ ID NO:6. The pTnMCS vector contains an avian optimized polyA sequence operably-linked to the transposase gene. The avian optimized polyA sequence contains approximately 40 nucleotides that precede the A nucleotide string.

Bp 1 – 130 Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp1-130

30 Bp 133 – 1777 CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems) bp 229-1873

Bp 1783 – 2991 Transposase, from Tn10 (GenBank accession #J01829) bp 108-1316

Bp 2992 – 3344 Non coding DNA from vector pNK2859

Bp 3345 – 3387 Lambda DNA from pNK2859

Bp 3388 – 3457 70 bp of IS10 left from Tn10

5 Bp 3464 – 3670 Multiple cloning site from pBluescriptII sk(-), thru the XmaI site bp 924-718

Bp 3671 - 3715 Multiple cloning site from pBluescriptII sk(-), from the XmaI site thru the XhoI site. These base pairs are usually lost when cloning into pTnMCS bp 717-673

10 Bp 3716 – 4153 Multiple cloning site from pBluescriptII sk(-), from the XhoI site bp 672-235

Bp 4159 - 4228 70 bp of IS10 right from Tn10

Bp 4229 - 4270 Lambda DNA from pNK2859

Bp 4271 – 5114 Non-coding DNA from pNK2859

15 Bp 5115 - 7315 pBluescript sk (-) base vector (Stratagene, Inc.) bp 761-2961.

EXAMPLE 3

Gene Therapy to Treat Cancer in an Animal

Preparation of the transposon-based vector

20 The following genes were cloned into the transposon-based vector of the present invention: an SV40 promoter linked to the preprosequence of cecropin B together with either a gene of interest encoding Phor14:beta human chorionic gonadotropin (bHCG), a gene of interest encoding gonadotropin releasing hormone (GnRH):Phor 11, or a gene of interest encoding GnRH:Phor 14, each linked to a 25 cecropin B poly A. The Phor peptides are lytic peptides. In this manner, three different vectors were created: 1) pTnPhor14:bHCG; 2) pTnGnRH:Phor 11; and, 3) pTnGnRH:Phor 14. The base vector used was pBTnLac. The SV40 promoter is a constitutive promoter expressed at a moderate level. The cecropin B prepro peptide was selected to permit a peptide to be transported out of the cell. The cecropin B poly 30 A was selected to terminate mRNA synthesis. These vectors in turn stimulate production of the fusion peptides, GnRH:Phor 11 (SEQ ID NO:44), GnRH:Phor 14 (SEQ ID NO:45), and Phor14:bHCG (SEQ ID NO:46). These transposon-based vectors were designed to provide an alternative to conventional chemotherapy in animals with tumors that express a receptor for luteinizing hormone (LH) or GnRH at

their surface, for example prostatic, ovarian, breast, pancreatic and some small cell lung carcinomas.

Mice, fish, cats and dogs have received these compositions without any adverse side effects. Temporary sterility was induced in these animals as evidenced
5 by a disrupted reproductive cycle.

The goal of this gene therapy was to administer the transposon-based vector complexed with a transfecting agent to the animal through any desired route (for example intravenous, intraperitoneal, intraarterial, or intramuscular) so that the animal makes the fusion protein. Next, the cells of the animal that expressed a receptor for
10 LH or GnRH recognized and bound the LH or GnRH component of the fusion peptide and delivered the fusion peptide containing the lytic peptide component to the cell, eventually resulting in lysis of the cell.

This approach to gene therapy permits very specific targeting of cells for destruction since cells expressing receptors specific for a ligand are affected without
15 affecting other cells as in conventional chemotherapy or radiotherapy. Further, this approach permits sustained delivery of the fusion peptides over time since the transgene is stably incorporated.

Use of the transposon-based vector in gene therapy for treatment of cancer in a dog
20 and in a cat

A dog with breast cancer metastasized throughout the body was treated. An aged female retriever of about 85 pounds body weight was diagnosed with widespread inflammatory mammary carcinoma. The initial diagnosis was confirmed with a skin biopsy of nodular lesions on the left lateral chest wall. The biopsy demonstrated
25 tumor nodules surrounded by fibrous tissue and tumor emboli within dermal lymphatic vessels. The tumor was composed of large, irregular, darkly blue stained cells with nuclear atypia and a high mitotic rate. The dog was administered the genetic constructs encoding for SEQ ID NO: 45 and SEQ ID NO: 46, i.v., at a dose of 50 ug of each construct in about 150 ul of transfection reagent (Superfect). Ten
30 days later the dog suddenly died. Within three hours, the skin lesion near the original biopsy site was removed and fixed. Histological analysis of sections from this biopsy revealed advanced and severe necrosis of the tumor cells both within the fibrous lesions and within the lymphatic vessels. Adjacent, normal non-neoplastic cells and tissues were non-necrotic. The death of tumor cells was estimated at more than 90%.

A cat with breast cancer metastasized throughout the body was treated. The cat was diagnosed with widespread inflammatory mammary carcinoma. The initial diagnosis was confirmed with a skin biopsy of nodular lesions. The cat was administered the genetic constructs encoding for SEQ ID NO: 45 and SEQ ID NO: 5 46, i.v. at a dose of 50 ug of each construct in about 150 ul of transfection reagent (Superfect). A subsequent histological analysis of sections from this biopsy revealed advanced and severe necrosis of the tumor cells both within the fibrous lesions and within the lymphatic vessels. Adjacent, normal non-neoplastic cells and tissues were non-necrotic.

10 The results demonstrate the efficiency of the gene therapy method of the present invention to treat cancer in an animal.

EXAMPLE 4

Development of a vector for tissue-specific insulin gene incorporation into animal 15 liver.

Figure 6 shows a scheme of a pTnMod-based vector for targeting an insulin gene into the liver. Using transposase (ATS) under control of liver-specific promoter, such as liver glucose-6-phosphatase (G6P) promoter, allows for tissue-specific incorporation of the insulin gene in the liver. The insulin gene is also placed under 20 control of a glucose-6 phosphatase (G6P) promoter.

The G6P promoter is cloned from rat genomic liver DNA. Rat genomic liver DNA is prepared according to procedures known to one of ordinary skill in the art. The promoter is cloned by amplifying the gene sequence using specific primers in a PCR reaction using methods known to one of ordinary skill in the art.

25 Alternatively, rat G6P promoter sequence is deduced from rat G6P gene untranslated upstream region provided in GenBank accession number U57552.1 and a corresponding synthetic oligonucleotide is prepared by methods known to one of ordinary skill in the art, preferably by any one of a number of commercial suppliers of synthetic oligonucleotides.

30 The gene encoding human proinsulin is amplified from human cDNA according to methods known in the art, for example, by using PCR with the primers specific for a proinsulin gene sequence, which is shown in SEQ ID NO:29. Briefly, total mRNA is isolated from a human pancreas according to procedures standard in the art. This mRNA is used to produce cDNA according to procedures standard in the

art. Alternatively, the promoter sequence is amplified by PCR from a commercially available human pancreatic cDNA library, such as the one supplied by Clontech Laboratories, Inc. under 2000 catalog number 7115-1, Human Pancreas QUICK-Clone cDNA.

5 Proinsulin is PCR-amplified from cDNA using specific primers designed in accordance with the proinsulin DNA sequence. The gene encoding proinsulin is cloned into the multiple cloning site (MCS) of pGWiz downstream of the G6P promoter sequence and upstream of the polyadenylation sequence (polyA).

10 Each of the identified components is sequenced, and cloned into pTnMod according to the scheme shown in Figure 6. Transposase (ATS) of the pTnMod vector is also placed under the control of the G6P promoter, which is obtained as described above, by subcloning G6P promoter sequence upstream of the pTnMod transposase sequence. Insertion sequences are denoted IS.

15 Any other desired components are prepared and incorporated into the vector by methods known to one of ordinary skill in the art. This vector is termed pTnModIns. Sufficient amounts of substantially pure TnModIns DNA are prepared using methods common in the art.

EXAMPLE 5

20 *Treatment of rats with the vector for tissue-specific insulin gene incorporation*

Diabetic rats are obtained made diabetic by administering the drug streptozotocin (Zanosar; Upjohn, Kalamazoo, MI) at approximately 200 mg/kg.

25 The rats are bred and maintained according to standard procedures. pTnModIns DNA, an appropriate carrier, and, optionally, a transfection agent, are injected into rats' singhepatic (if using G6P) artery with the purpose of stable transformation. Incorporation of the insulin gene into the rat genome and levels of insulin expression are ascertained by a variety of methods known in the art. Blood and tissue samples from live or sacrificed animals are tested. A combination of PCR, 30 Southern and Northern blots, in-situ hybridization and related nucleic acid analysis methods are used to determine incorporation of the vector-derived proinsulin DNA and levels of transcription of the corresponding mRNA in various organs and tissues of the rats. A combination of SDS-PAGE gels, Western Blot analysis, radioimmunoassay, and ELISA and other methods known to one of ordinary skill in the art are used to determine the presence of insulin and the amount produced.

Additional transfections of pTnModIns are used to increase protein expression if the initial amounts of the expressed insulin are not satisfactory, or if the level of expression tapers off. The physiological condition of the rats is closely examined post-transfection to register positive or any negative effects of the gene therapy.

5 Animals are examined over extended periods of time post-transfection in order to monitor the stability of gene incorporation and protein expression.

EXAMPLE 6

Intracardiac Injection of a Transposon-based Vector for Gene Therapy and 10 Production of Transgenic Quail

Direct cardiac injection coupled with a transposon-based vector was used to provide direct incorporation into either liver, oviduct, or ovaries and progenitor cells of each. The technique may also be used to transform the progenitor cells (spermatogonia) in the testes to give rise to transgenic sperm. Stable incorporation of 15 the vector DNA in progenitor cells results in long term production of transgenic liver cells, ova and oviduct cells, including tubular gland cells, and sperm; presumably for the life of the bird.

Five Japanese quail from Louisiana State University (LSU) stock and five from Bull Run stock were anesthetized and injected in the left ventricle of the heart in 20 with 20 µg of the transposon-based vector and transfection reagent in a total volume of 0.35 ml. A needle approximately 5/8 inches (25 gauge) in length was used for injections of LSU quail. A needle approximately 1 inch (22 gauge) in length was used for injections of Bull Run quail. The needles were connected to a 1ml tuberculin syringe containing the transfection mixture. Birds were held in the hand with the keel 25 up. Feathers in the area of the left breast were grasped and a few down feathers removed over the injection site. The area sprayed with ethanol.

The injector placed his left hand over the bird with the tip of the forefinger placed on the anterior tip of the keel. The thumb was used to palpate the triangle-shaped, posterior end of the caudolateral process of the sternum. The caudolateral 30 process was followed forward to where it joined the body of the sternum. This marked the U shaped bony border of the lateral notch. The U of the lateral notch is formed by the thoracic process and the caudolateral process. While maintaining the thumb in the lateral notch, an imaginary line was drawn straight down from the forefinger. Another imaginary line was drawn at the angle of the caudolateral process

from the tip of the thumb forward. The site where the needle was placed was the intersection of these lines. This is approximately 2cm towards the bird's head from the tip of the thumb.

5 The needle and syringe were held parallel to the table. The needle was inserted into the superficial pectoralis muscle. Without completely withdrawing the needle, it was repositioned slightly to one side or the other until an intercostal space was found.

10 The needle was placed into the left breast muscle at about a 45° angle. When the needle was about halfway in, the needle hit the sternum. Next, the needle was partially removed and repositioned at a steeper angle until the sternum was no longer encountered. At this angle the needle dropped into the left ventricle of the heart. A flash of blood appeared in the syringe and pulsed in the hub at the rate of the heartbeat. The plunger on the syringe was slowly depressed. If there was an air bubble above the solution inside the syringe, the plunger was stopped before the air 15 was pushed out into the blood. The needle was removed and disposed in a biohazard sharps container. The bird was returned to its cage and monitored for any signs of distress. (See A Color Atlas of Avian Anatomy. John McLelland. W.B. Saunders Company, 1991 for view of the anatomy of this area).

20 The vector CMVp/pp/HC/ProLys/LC/CPA (SEQ ID NO: 47), which encodes monoclonal antibody RM-2, was injected into the left ventricle of female Japanese quail. These birds were held for 2 days post-injection and sacrificed by cervical dislocation. Immediately after sacrifice, the visceral cavity of each bird was opened and a piece of liver, ovary, and oviduct was removed. For oviduct, a section from the magnum was removed and scissors were used to make a longitudinal cut that opened 25 the tube and allowed it to lay flat. Once the luminal folds were exposed, the tops of the folds were removed and used for tissue extraction. Using the tops of these folds ensured that the most abundant cell type was the tubular gland cell.

30 Approximately 5 mg of each tissue type was used for genomic DNA isolation using a Qiagen Genomic DNA isolation kit. DNA was quantified and used in a PCR reaction with primers HC-1 and HC-4 that amplify a section of the human IgG heavy chain. The vector used for these injections served as a positive control in the PCR reactions. One LSU bird (Bird 2211) and one Bull Run bird (bird 2895) did not receive an injection and were used as negative controls.

In order to determine if gene incorporation (transposition) was occurring, instead of maintenance of the vector, PCR was conducted using a primer anchored in the gene of interest and the transposase. The result was a greatly reduced PCR reaction when compared to the PCR result from the heavy chain indicating the vector 5 was being destroyed while the target gene was being maintained in the recipient chromosome.

PCR was conducted on the liver of quail injected in the left ventricle with a transposon-based vector encoding for the monoclonal MCS(CMVP/PP/HC/ProLys/LC/CPA) SEQ ID NO:48. Primers designed to the 10 heavy chain of the monoclonal resulted in the correct PCR fragment in all of the injected birds and the positive vector control. All control birds, PCR controls, and kit controls resulted in a negative PCR reaction. This PCR reaction proved DNA uptake by the liver in birds that received a cardiac injection. One bird was slightly weaker on an oviduct sample. These results clearly demonstrate DNA presence in high 15 quantities two days post-cardiac injection.

In order to determine if transposition occurred, the same quantity of DNA was used in the PCR reaction containing primers HC 1 and HC 4 as was used in the PCR reaction containing primers mATS3'F and mATS5'R (these primers amplify a segment of DNA within the transposase). If no transposition occurred, then the bands 20 in each reaction would be very similar in intensity. If transposition occurred, then the bands would not be similar. In order to make sure there was not a problem with the buffer chosen, 3 buffers from an optimization kit were used. Due to the band intensity from the initial PCR, the number of cycles was decreased from 45 to 30 in order to detect any small differences that might occur.

25 As seen in the transposition PCR, the band corresponding to the transposase was present, but at a concentration much less than the heavy chain fragment that was amplified. The results also demonstrate that the transposase is degraded which the gene encoding for the heavy chain is stably incorporated. This indicates that transposition has occurred and that the majority of the amplicon was due to copies 30 integrated into the quail genome. Using such a delivery system combined with a transposon-based vector allows rapid expression of a gene for protein production in the liver or oviduct, or allows production of transgenic hens and roosters equivalent to G2 offspring if a traditional route of transfecting one animal and crossing to increase gene copy number is used.

EXAMPLE 7

Intracardiac Injection of a Transposon-based Vector for Gene Therapy and Production of Proinsulin in Transgenic Chickens

A total of 6 mature white leghorn hens were anesthetized and injected into the 5 left ventricle of the heart with 1 ml total volume (consisting 50 µg of DNA, 150 µl of Superfect supplemented with HBSS to 1 ml). The methods are similar to those described in the preceding example for quail.

Briefly, a needle approximately 1 inch (22 gauge) in length was used for 10 injections of chickens. The needles were connected to a 1ml tuberculin syringe containing the transfection mixture. Birds were held at the base of the wings on a table. Feathers in the area of the feather track about halfway down the breast were grasped and a few down feathers removed over the injection site. The area sprayed with ethanol.

The injector placed his left hand over the bird with the tip of the forefinger 15 placed on the anterior tip of the keel. The thumb was used to palpate the triangle-shaped, posterior end of the caudolateral process of the sternum. The caudolateral process was followed forward to where it joined the body of the sternum. This marked the U shaped bony border of the lateral notch. The U of the lateral notch is formed by the thoracic process and the caudolateral process. While maintaining the 20 thumb in the lateral notch, an imaginary line was drawn straight down from the forefinger. Another imaginary line was drawn at the angle of the caudolateral process from the tip of the thumb forward. The site where the needle was placed was the intersection of these lines. This is approximately 2cm towards the bird's head from the tip of the thumb.

25 The needle and syringe were held parallel to the table. The needle was inserted into the superficial pectoralis muscle. Without completely withdrawing the needle, it was repositioned slightly to one side or the other until an intercostal space was found.

30 The needle was placed into the left breast muscle at about a 45° angle. When the needle was about halfway in, the needle hit the sternum. Next, the needle was partially removed and repositioned at a steeper angle until the sternum was no longer encountered. At this angle the needle dropped into the left ventricle of the heart. A flash of blood appeared in the syringe and pulsed in the hub at the rate of the heartbeat. The plunger on the syringe was slowly depressed. If there was an air

bubble above the solution inside the syringe, the plunger was stopped before the air was pushed out into the blood. The needle was removed and disposed in a biohazard sharps container. The bird was returned to its cage and monitored for any signs of distress. (See A Color Atlas of Avian Anatomy. John McLelland. W.B. Saunders Company, 1991 for view of the anatomy of this area).

5 The vector SEQ ID NO: 49 encoded for chicken ovalbumin::ent Tag::proinsulin fusion protein (Vector: pTnMCS (ChOVep/OVg'::ent/pro-ins/syn poly A) (Clone MCS6). Twenty-four hours post-injection, two birds were sacrificed and liver, ovary and oviduct tissue was removed from each bird. Genomic DNA was
10 extracted from each tissue as described previously. PCR was conducted and a sample of that reaction was electrophoresed on a 2% gel. The remaining 4 chickens are laying eggs that are currently being evaluated for the presence of the fusion protein.

15 PCR was conducted on DNA isolated from the liver, oviduct and ovary of two chickens (2004, 2005) injected in the left ventricle with a transposon-based vector encoding for ovalbumin::ent Tag::proinsulin fusion protein SEQ ID NO:49. The result was a positive PCR reaction from each DNA sample, regardless of the tissue type and the vector control. All kit and PCR controls were negative indicating no contamination had occurred. The results show band amplified by PCR that indicate that the gene encoding for proinsulin is present in the liver, ovary and oviduct of each
20 of the two chickens examined.

SEQ ID NO: 49 pTnMCS(Chicken OVep+OVg'+ENT+proins+syn polyA) was constructed as follows:

Bp 1 – 3670 from-vector pTnMCS, bp 1 - 3670

25 Bp 3676 – 4350 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1 bp 1-675

Bp 4357 – 5692 Chicken Ovalbumin promoter taken from GenBank accession # J00895-M24999 bp 1-1336

Bp 5699 – 6917 Chicken Ovalbumin gene from GenBank Accession # V00383.1 bp
30 2- 1220. (This sequence includes the 5'UTR, containing putative cap site, bp 5699-5762.)

Bp 6924 - 7073 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7074 - 7334 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 7335 - 7379 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 7380 - 7731 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

5 Bp 7733 - 11332 from vector pTnMCS, bp 3716 - 7315

All patents, publications and abstracts cited above are incorporated herein by reference in their entirety, including U.S. provisional patent applications serial numbers 60/532,504, 60/565,371 and 60/592,098, and PCT patent applications 10 PCT/US03/41261, PCT/US03/41269, and PCT/US03/41335. It should be understood that the foregoing relates only to preferred embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the present invention as defined in the following claims.

15

TABLE 1

	Promoter	Ref.	Function/comments
Reproductive tissue			
testes, spermatogenesis	SPATA4	1	constitutive 30 d after birth in rat URE, Upstream Regulatory Element
placenta, glycoprotein breast epithelium and breast cancer	ERVWE1	2	is tissue spec. enhancer
prostate	mammaglobin	6	specific to breast epithelium and cancer
prostate	EPSA	17	enhanced prostate-specific antigen promoter
testes	ATC	25	AlphaT-catenin specific for testes, skeletal, brain cardiomyocytes
prostate	PB	67	probasin promoter
Vision			
rod/cone	mCAR	3	cone photoreceptors and pinealocytes
retina	ATH5	15	functions in retinal ganglia and precursors
eye, brain	rhodopsin	27	
kerocytes	keratocan	42	specific to the corneal stroma
retina	RPE65	59	
Muscle			Tissue Factor Pathway Inhibitor – low level expression in endothelial and smooth muscle cells of vascular system
vascular smooth muscle cardiac specific	TFPI	13	
cardiac	MLC2v	14, 26	ventricular myosin light chain BMP response element that directs cardiac specific expression
skeletal	CAR3	18	high level, muscle spec expression
skeletal	C5-12	22	to drive target gene
smooth muscle	<i>AdmDys</i> , AdmCTLA4lg	32	muscle creatine kinase promoter
smooth muscle	PDE5A	41	chromosome 4q26, phosphodiesterase use intronic splicing elements to restrict expression to smooth
skeletal	AlphaTM	45	muscle vs skeletal
skeletal	myostatin	48	fiber type-specific expression of myostatin
Endocrine/nervous			
glucocorticoid neuroblastoma	GR 1B-1E	4, 12	glucocorticoid receptor promoter/ all cells
	M2-2	8, 36	M2 muscarinic receptor
brain	Abeta	16	amyloid beta-protein; 30 bp fragment needed for PC12 and glial cell expression
brain	enolase	21	neuron-specific, high in hippocampus, intermediate in cortex, low in cerebellum
synapses	rapsyn	29	clusters acetylcholine receptors at neuromuscular junction
neuropeptide precursor	VGF	39	express limited to neurons in central and peripheral nervous system and specific endocrine cells in adenohypophysis, adrenal medulla, GI tract and pancreas
mammalian nervous system central and peripheral noradrenergic neurons	BMP/RA	46	use of methylation to control tissue specificity in neural cells.
	Phox2a/Phox2b	47	regulation of neuron differentiation

brain	BAI1-AP4	55	spec to cerebral cortex and hippocampus
Gastrointestinal			
UDP glucuronosyltransferase	UGT1A7	11	gastric mucosa
	UGT1A8	11	small intestine and colon
	UGT1A10	11	small intestine and colon
colon cancer	PKCbetaII	20	Protein kinase C betaII (PKCbetaII); express in colon cancer to selectively kill it.
Cancer			
tumor suppressor 4.1B	4.1B	5	2 isoforms, 1 spec to brain, 1 in kidney
nestin	nestin	63	second intron regulates tissue specificity
cancer spec promoter	hTRT/hSPA1	68	dual promoter system for cancer specificity
Blood/lymph system			
Thyroid	thyroglobulin	10	Thyroid spec. -- express to kill thyroid tumors
Thyroid	calcitonin	10	medullary thyroid tumors
Thyroid	GR 1A	12	regulation controlled by DREAM
thyroid	thyroglobulin	50	transcriptional repressor
arterial endothelial cells	ALK1	60	activin receptor-like kinase
Nonspecific			
RNA polymerase II		7	
gene silencing	Gnasx1, Nespas	31	
beta-globin	beta globin	53	
Cardiac	M2-1	8	M2 muscarinic receptor
Lung	hBD-2	19	IL-17 induced transcription in airway epithelium
pulmonary surfactant protein	SP-C	62	Alveolar type II cells
ciliated cell-specific promoter	FOZJ1	70	use in ciliated epithelial cells for CF treatment
surfactant protein expression	SPA-D	73	Possible treatment in premature babies
Clara cell secretory protein	CCSP	75	
Dental			
teeth/bone	DSPP	28	extracellular matrix protein dentin sialophosphoprotein
Adipose			
adipogenesis	EPAS1	33	endothelial PAS domain -- role in adipocyte differentiation
Epidermal			
differentiated epidermis	involucrin	38	
desmosomal protein	CDSN	58	stratum granulosum and stratum corneum of epidermis
Liver			
liver spec albumin	Albumin	49	
serum alpha-fetoprotein	AFP	56	liver spec regulation

References

1. Biol Pharm Bull. 2004 Nov;27(11):1867-70
2. J Virol. 2004 Nov;78(22):12157-68
- 5 3. Invest Ophthalmol Vis Sci. 2004 Nov;45(11):3877-84
4. Biochim Biophys Acta. 2004 Oct 21;1680(2):114-28
5. Biochim Biophys Acta. 2004 Oct 21;1680(2):71-82
6. Curr Cancer Drug Targets. 2004 Sep;4(6):531-42
7. Biotechnol Bioeng. 2004 Nov 20;88(4):417-25
- 10 8. J Neurochem. 2004 Oct;91(1):88-98
9. Curr Drug Targets Immune Endocr Metabol Disord. 2004 Sep;4(3):235-44
10. Toxicol Appl Pharmacol. 2004 Sep 15;199(3):354-63
11. J Immunol. 2004 Sep 15;173(6):3816-24
12. Thromb Haemost. 2004 Sep;92(3):495-502
13. Acad Radiol. 2004 Sep;11(9):1022-8
14. Development. 2004 Sep;131(18):4447-54
- 15 15. J Neurochem. 2004 Sep;90(6):1432-44
16. Mol Ther. 2004 Sep;10(3):545-52
17. Development. 2004 Oct;131(19):4709-23. Epub 2004 Aug 25
18. J Immunol. 2004 Sep 1;173(5):3482-91
19. J Biol Chem. 2004 Oct 29;279(44):45556-63. Epub 2004 Aug 20
- 20 20. J Biol Chem. 2004 Oct 22;279(43):44795-801. Epub 2004 Aug 20
21. Hum Gene Ther. 2004 Aug;15(8):783-92
22. Nucleic Acids Res. 2004 Aug 09;32(14):4155-65. Print 2004
23. Mol Imaging. 2004 Apr;3(2):69-75
24. J Gene Med. 2004 Aug;6(8):906-12
- 25 25. J Biol Chem. 2004 Oct 1;279(40):42182-91. Epub 2004 Jul 28
26. Mol Cell Biol. 2004 Aug;24(16):7188-96
27. Nat Genet. 2004 Aug;36(8):894-9. Epub 2004 Jul 25
28. Gene Ther. 2004 Oct;11(19):1453-61
29. J Biol Chem. 2004 Sep 24;279(39):40946-53. Epub 2004 Jul 15
- 30 30. Brain Res Mol Brain Res. 2004 Jul 26;126(2):173-80
31. J Invest Dermatol. 2004 Aug;123(2):313-8
32. Cell Mol Neurobiol. 2004 Aug;24(4):517-33
33. Int J Impot Res. 2004 Jun;16 Suppl 1:S8-S10
34. Invest Ophthalmol Vis Sci. 2004 Jul;45(7):2194-200
- 35 35. J Biol Chem. 2004 Aug 27;279(35):36660-9. Epub 2004 Jun 11
36. Brain Res Mol Brain Res. 2004 Jun 18;125(1-2):47-59
37. Brain Res Mol Brain Res. 2004 Jun 18;125(1-2):29-39
38. Am J Physiol Cell Physiol. 2004 Oct;287(4):C1031-40. Epub 2004 Jun 09
39. Am J Physiol Lung Cell Mol Physiol. 2004 Dec 3; [Epub ahead of print]
- 40 40. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2003 Nov;19(6):601-3
41. J Biol Chem. 2004 Aug 6;279(32):33114-22. Epub 2004 Jun 04
42. Brief Funct Genomic Proteomic. 2004 Feb;2(4):344-54
43. FEBS Lett. 2004 May 21;566(1-3):87-94
44. Biochem Biophys Res Commun. 2004 Jun 4;318(3):773-85
- 45 45. J Invest Dermatol. 2004 Mar;122(3):730-8
46. Mol Vis. 2004 Mar 26;10:208-14
47. Circ Res. 2004 Apr 30;94(8):e72-7. Epub 2004 Apr 01
48. Lab Invest. 2004 Dec;84(12):1581-92
49. Prostate. 2004 Jun 1;59(4):370-82
- 50 50. Cancer Res. 2004 Jan 1;64(1):363-9
51. Mol Ther. 2003 Oct;8(4):637-45
52. Front Biosci. 2003 May 01;8:d751-64

75. Am J Respir Cell Mol Biol. 2002 Aug;27(2):186-93

Table 2

Gene-protein target*	Cellular function	Type of cancer tested
<i>B-raf</i>	Serine/threonine kinase	Malignant melanoma
Nox1	Superoxide-generating oxidase	Transformed MRK cells ^a
FAS/Her2	Fatty acid synthase	Breast-MDA-MB-231
Cyclin E	Cell-cycle control	Hepatocarcinoma
Hec1	Chromosomal segregation	
Gp210	Nuclear pore assembly	Adenocarcinoma (HeLa cells)
c-Kit	Signal transduction	Gastrointestinal
MDR	Multi-drug resistance	Adenocarcinoma (HeLa cells)
<i>bcl-2</i>	Antiapoptotic	Esophageal adenocarcinoma
<i>livin</i>	Antiapoptotic	Adenocarcinoma
<i>survivin</i>	Antiapoptotic	Adenocarcinoma (HeLa cells)
Philadelphia chromosome		Chronic myeloid leukemia
Ribonucleotide reductase	Gemcitabine resistance	Hepatic metastasis
Rho C	Cell motility	Metastasis

^aNormal rat kidney cells.

*Genes are written in italics and lower case letters while proteins begin with a capital letter and are written in roman letters.

Table 3

Genetic Disorder	Gene	Chromosome
Hemophilia	CRHR1	
Depression	SCID-X1	X
SCIDS	ESR1	
Breast Cancer	BRCA1	
	BRCA2	
	p53	
Lupus (SLAM/C D2	Sles1	
	Sles2	
	Sles3	
	Sles4	
Colon Cancer	15-PGDH	
	MSH2	2
	MSH6	2
	MLH1	3
Crohn's Disease	CD 19	16
	sialophorin	
	CD11 integrin	
	IL-4	
Cystic Fibrosis	CFTR	
Type I Diabetes	IDDM1	6
	IDDM2	11
	GCK (glucokinase)	7
Glucose/Galactose	SGLT1	22
Malabsorption (CGM)		
Pancreatic Cancer	DPC4 (Smad4)	18
	p53	
	Rb	
Wilson's disease	ATP7B	13
Zellweger syndrome	PXR 1	12
Sickle Cell Anemia	HBB	11p15.4
Burkitt Lymphoma	Myc	8
Gaucher disease	glucocerebroside	
Hemophilia A	HEMA (Factor VIII)	X
Chronic Myeloid leukemia	ABL (9) BCR (22)	9/22 exchange
Niemann-Pick Type A, B or C	NP-C	18
Hemoglobinuria (PNH)	PIG-A	X
Porphyria		
Thalassemia alpha	HBA1	16
	HBA2	16
Thalassemia beta	HBB	11
Small cell lung carcinoma		3
Melanoma	CDKN2	9
Multiple endocrine neoplasia	MEN1	11
Neurofibromatosis	NF-2	22
Li-Fraumeni syndrome	p53	17
	Rb	13
Polycystic Kidney Disease	PKD1	16
Prostate cancer	HPC1	1
Harvey Ras oncogene	Ras	11
Tuberous sclerosis	TSC1	9
	TSC2	16

Von-Hippel Lindau	VHL	3
Deafness	Cx26	13q11-12
Pendred syndrome	PDS	7
Best disease	VMD2	11
Glaucoma	GLC1A	1
gyrate atrophy	OAT	10
Rett syndrome	MeCP2	Xq28
Congenital adrenal hyperplasia	CYP21P	6
Adrenoleukodystrophy	ALD	
AI polyglandular syndrome I	AIRE	21
Cockayne syndrome Type I	CSA	5
Cockayne syndrome Type II	CSB	
Dlastrophic dysplasia	DTD	5
Ataxia telangiectasia	ATM	11
Atherosclerosis	Apo E	19
Long QT syndrome	LQT1	11
Williams Syndrome	LIM kinase, elastin	7
Asthma		5, 6, 11, 14, 12
DiGeorge syndrome		22
Hyper-IgM	TNFSF5	Xq26
Severe Combined Immunodeficiency Disease (SCID)	IL2RG	X
	JAK3	19
	ADA	20
Alport Syndrome	COL4A5	X
5-alpha reductase		5
Achondroplasia	FGFR3	4
Familial ALS	SOD1	21
Charcot-Marie-Tooth disease Type 1A	PMP22	17
Charcot-Marie-Tooth disease Type 1B		X
Dejerine-Sottas Syndrome		
Duchenne muscular dystrophy	dystrophin	X
Ellis-van Creveld syndrome	EVC	4
Fibrodysplasia Ossificans Progressiva	NOG	
	BMP	
Marfan Syndrome	FBN1	15
Myotonic dystrophy	myotonic dystrophy	19
Fragile X	FMR1	X
PWS	SNRPN	15
Waardenburg syndrome	Pax3	2
Werner disease	SGS1	8
Alzheimer disease	PS1	14
	PS2	1
Angelman syndrome	deletion 15q11q13	15
	UBE3A	
Essential tremor	ETM1	3
	ETM2	2
Familial Mediterranean fever	FMF	16
Friedreich's ataxia	YFH1	
Huntington's disease	HD	4
Maple Syrup Urine Disease	BCKDH complex	
Parkinson's	Alpha-synuclein	4
Refsum disease	PAHX	10
Spinal Muscular Atrophy	SMN1	5
	SMN2	
spinocerebellar ataxia	SCA1	6

Tangier Disease	ABC1	9q31
Tay-Sach's	HEXA	15
Obesity	leptin	7
AAT	SERPINA 1	14
Hemochromatosis	HFE (mutation C282Y) HFW (mutation H63D)	

CLAIMS

We claim:

5 1. A composition comprising a transposon-based vector comprising :

- a) a gene operably linked to a first promoter, the gene encoding for a bacterial transposase; and,
- b) one or more genes of interest operably-linked to one or more additional promoters,

10 wherein the one or more genes of interest and their operably-linked promoters are flanked by transposase insertion sequences recognized by the bacterial transposase, wherein the first promoter and the one or more additional promoters are cell-specific promoters or constitutive promoters.

15 2. The transposon-based vector of claim 1, further comprising an isolated polyA nucleotide sequence located 3' to the one or more genes of interest.

3. The isolated polyA nucleotide sequence of claim 2, wherein the isolated polyA nucleotide sequence is optimized for production of a protein, peptide or nucleic acid encoded by the one or more genes of interest.

20 4. The transposon-based vector of claim 1, wherein the one or more genes of interest code for a protein, a peptide or a nucleic acid.

25 5. The transposon-based vector of claim 1, wherein the one or more gene of interest encodes for a nucleic acid which inhibits transcription.

6. A composition comprising an isolated polynucleotide sequence comprising:

30 a) one or more genes of interest operably-linked to one or more promoters;

- b) a poly A nucleotide sequence located 3' to the one or more genes of interest; and,

c) transposase insertion sequences recognized by a bacterial transposase,

5 wherein the one or more genes of interest and their operably-linked promoters are flanked by the transposase insertion sequences and the one or more additional promoters are cell-specific promoters or constitutive promoters.

7. The isolated polynucleotide sequence of claim 6, wherein the one or more genes of interest code for a protein, a peptide or a nucleic acid.

10

8. An animal or a human comprising the isolated polynucleotide sequence of claim 6.

15

9. The animal of claim 8, wherein the animal is a bird or a mammal.

10. An egg produced by the bird of claim 9.

11. Milk produced by the mammal of claim 9.

20

12. A cell comprising the isolated polynucleotide sequence of claim 6.

13. A method of providing gene therapy to an animal or a human comprising administering to the animal or the human the transposon-based vector of Claim 1.

25

14. The method of claim 13, wherein the one or more additional promoter is a cell specific promoter.

30

15. The method of claim 13, wherein the gene of interest codes for production of a protein, peptide or nucleic acid.

16. The method of claim 13, further comprising a polyA sequence located 3' to the one or more genes of interest.

17. The method of claim 13, wherein the gene therapy comprises production of a protein, peptide or nucleic acid encoded by the one or more genes of interest in the animal or the human.

5 18. The method of claim 13, wherein the administration is effective to treat a disease or a condition.

19. The method of claim 13, wherein the administration of the transposon-based vector results in a transfection rate of at least 40%.

10 20. The method of claim 13, wherein the administration occurs through the vascular system.

21. An animal produced by the method of claim 13.

15 22. Use of the composition of any one of claims 1-7, in the preparation of a medicament useful for providing gene therapy to an animal or human following administration of an effective amount of the composition to the animal or the human.

20 23. The use of claim 22, wherein the gene therapy treats a disease or a condition in the animal or the human.

FIGURE 1



FIGURE 2

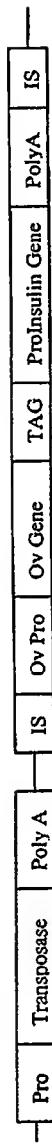


FIGURE 3

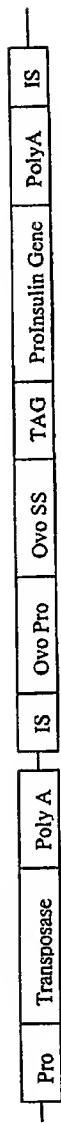
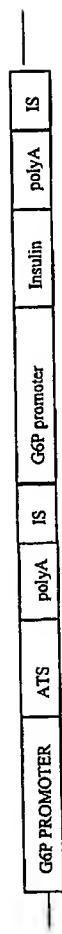


FIGURE 4

IS	Tet ₄ Pro	Ovgen	Pro	Ovotrans	Pro	Ovomucin	IS
----	----------------------	-------	-----	----------	-----	----------	----

Figure 5.



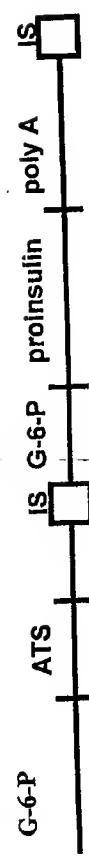


Figure 6

Figure 7



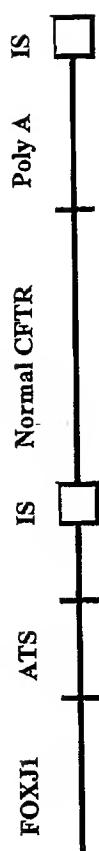


Figure 8



Figure 9

APPENDIX

2461 tgccaaattc tattgtataa atctcgctct aaaggccgaa aaaatcagcg ctcgacacgg
 2521 actcattgtc accacccgtc acctaaaatc tactcagcgt cggcaaaagg gccatgggt
 2581 ctagcaacta acttacctgt tgaattcga acacccaaac aacttgtaa tatctattcg
 2641 aagcgaatgc agattgaaga aacccctccga gacttgaaa gtcctgccta cggactaggc
 5 2701 ctacgcata gccgaacgg cagctcagag cggtttgata tcatgcgt aatccgcctg
 2761 atgcctcaac taacatgtt gttgcgggc gttcatgcg agaaaacaagg ttggacaag
 2821 cactccagg ctaacacagt cagaatcga aacgtactct caacagttcg cttaggcatt
 2881 gaagtttgc ggcattctgg ctacacaata acaaggaaag acttactcgt ggctgcaacc
 2941 ctactagtc aaaattttt cacacatgtt taegctttgg gaaattatg agggatcgc
 10 3001 tctagagcga tcggggatct cggggaaagg gttggtgacc aagggtgcct ttatcatca
 3061 cttaaaaat aaaaacaat tactcagtgc ctgttataag cagcaatcaa ttatgattga
 3121 tgctcacatc acacaaaaaa ctgatattaac aaatggttgg tctgccttag aaagtatata
 3181 tgaacattat ctgattata ttattgataa taataaaaaac cttatcccta tccaagaagt
 3241 gatgcctatc attgggttgg atgacttga aaaaatttag ccttgaatac attactggta
 15 3301 aggtaaacgc attgtcagc aaaaaatgtacc aagagaacca acttaaaatgc ttcctgacgg
 3361 aatgttattat ctcgttgcct ctgagacty atgaatcccc taatgattt ggtaaaaatc
 3421 attaagttaa ggtggataca cattttgtca tatgatcccg gtaatgttag ttagctact
 3481 cattaggcac cccaggctt acactttatg ctccggcgc gtatgttg tgaaatttg
 3541 agcggataac aatttcacac agggaaacagc tatgaccatc attacgcacaa ggcgcacatt
 20 3601 aaccctcaact aaaaaggaa aagactggag ctccacccgc gtggcggccg ctctagaact
 3661 agtggatccc cggggtgcga ggaattcgat atcaagctt tcgataccgc tgacccgc
 3721 gggggggcccg gtaccaatt cggccctatag tgagtcgtat tacgcgcgt cactggccgt
 3781 cgttttacaa cgtcgtact gggaaaaccc tggcgttacc caacttatac gcttgcacgc
 3841 acatccccct tteccagct ggcgtatag cgaagaggcc cgcacccgatc gcccctccca
 25 3901 acatgttgcgc acctgtatc gcaatggaa attgttgcg ttaatattt gttaaaattc
 3961 gcgtttaat tttgttaat cagtcattt ttaaacaat aggccaaat cggcaaaatc
 4021 ctttataat caaaagaata gaccgagata ggggtttagt tggttccagt ttggacaacag
 4081 agtccactat taaaagaaacgt ggactccaaac gtcaaaaggc gaaaaacccgt ctatcaggc
 4141 gatggcccaac tactccggaa tcatatgaca agatgttgcat ccacccatc ttaatgatt
 30 4201 ttaccaaaaat cattaggaa ttcatcgttgc ttcagggtca acggaaatc acatccgtc
 4261 agggaaagtt atgtatgtt gttgtttaaa aacttactca atggctgtt atgcataatcg
 4321 caatacatgc gaaaacacta aaagagctt cggataaaaa aggccaaattt attgttattt
 4381 acggcggctt tttatttgc ttgaaagata aataaaaatag ataggtttta ttggaaageta
 4441 aatcttctt atcgttaaaaat atgccttctt gggttatcaa gagggttcatc atatttcgc
 35 4501 gaataacatc atttgggtac gaaataacta agcacttgc tcctgttac tcccctgac
 4561 ttgaggggtt aacatgaaagg tcatcgtatc caggataata atacagtaaa acgctaaacc
 4621 aataatccaa atccagccat cccaaatttg tagtgaatga ttataataa cagcaacac
 4681 taatgggcca ataacacccgg ttgcatttgc aagggtcacc aataatccct gtaaagcacc
 4741 ttgcgtatc ctctttgtt ggtatcactt cactccctgt aatgcaggta aagcgatccc
 40 4801 accaccagcc aaaaatattt aacacggaa aactaaccat ctttcagata taaacgctaa
 4861 aaaggcaaat gcactactat ctgcaataaa tccgagcaatc actggcgttt ttgcggccat
 4921 ttatgttgc ttccttgc cacaaggct tggaaatctc agtgtaaaag accaagaccc
 4981 gtaatgaaaaa gccaaccatc atgcattatca tcatcgtatc ttctgtatata gcaaccacacc
 5041 gtgtggatt ggctatcatc ggcgttcaat aataatcaac aaatggcattc gttaaaataag
 45 5101 ttagtatac cgtatcgat ttgttccctt tagtgggggt taattgcgcg cttggcgtaa
 5161 tcatggtcat agctgttcc ttgtgttaat ttgttccgc tcacaattcc acacaacata
 5221 cggccggaa gcataaaatgt taaagcttgc ggtgcataat gatgtgatcacta actcacatta
 5281 attcgttgc gtcacttgc cgttccatc tggggaaaccat tggcgttgc gtcgtatc
 5341 tgaatccggc aacgcgggg ggggggggtt ttgcgttattt ggcgttccgc cgttccctcg
 50 5401 ctactgact cgtcgccctc ggtcggttgc ctgcggcgag cggatcagc tcactcaag
 5461 gcgtaataac gtttatccatc agaatcggg gataacgcg gaaagaaatc gtgacaaaaa
 5521 gcccggaaa aggccaggaa cggtaaaaaag gcccgcgttgc tggcggtttt ccataggctc
 5581 cggcccccgtt acgagatc caaaaatcga cgttcaatc agagggtggcg aaacccgaca
 5641 gatgttgcataa gataccaggc gtttccccctt ggaagctccc tcgtcgctc tcctgttccg
 5701 accctggcc ttaaccgata cctgtccgc ttcttccctt cggaaacgcg ggcgttcc
 5761 catagctcact gtcgttagtta ttcgttgc tggtaggttgc ttgcgtccaa gctggctgt
 5821 gtgcacgaaac ccccccgttca gcccggccgc tggcccttgc cggtaacta tcgttcc
 5881 tccaaaccgg taagacacga cttatcgcca ctggcagatc ccactgttgc caggattac
 5941 agaggcggat atgttgcggg tgctacagat ttcttgcgtt ggtggcttca ctacggctac
 6001 actagaagga cagtattttg tatctgcgt ctgcgttgc cagttacctt cggaaaaaga
 6061 gttggtagct ttgtatccgg caaacaaacc accgttgcgttgc ggggtgggtt ttttttttgc
 6121 aacgcggcata ttacgcggcag aaaaaaaaggatc ttcgttgc ttttttgc ttttttgc
 6181 ggggttgcgttgc cttatcgatc cggtaatc cggtaaggaa ttttttgc ttttttgc
 6241 aaaaaggatct tcacccatc cttttttatc taaaatgtt gtttttgc ttttttgc
 6301 atatgtatc aacatgggtc tgacagtatc caatgccttca tcgttgcggc acctatctca
 6361 gcgatctgtc ttttttgc ttttttgc ttttttgc ttttttgc ttttttgc
 6421 atacgggggg gtttccatc tggcccccgttgc gtcgtatc taccgcgaga cccacgcgtca
 6481 cccggctccatc attatcgatc aataaaccat cccggccggaa gggccggcg cagaatgttgc

6541 cctgcaactt tatccgcctc catccagtctt attaattgtt gcccggaaagc tagagtaagt
 6601 agtgcgcag ttaataqttt gcgcacgtt gttgccattt ctacaggcat cgtgggtca
 6661 cgctcgctgt ttgttatggc ttcatcgcc tccgggtccc aacgatcaag gcgagttaca
 6721 tgcattccca tgggtgtcaa aaaaagcggtt agctccttcg gtcctccat cgttgtcaga
 5 6781 agtaagtgg cccgactgtt atcaactcatg gttatggcag cactgcataa ttctcttact
 6841 gtcattccat cccgtaaatg ctttctgtg actggtagt actcaaccaa gtcattctga
 6901 gaatagtgtt tgcggcggacc gagttgtctt tgcggcgctt caataccggta taataccgc
 6961 ccacatagca gaactttaaa agtgcgtatc attggaaaac gttcgtccgg gcgaaaactc
 7021 tcaaggatct tacccgtt gatgtccatg tgcgtgtaa ccactcgtc acccaactga
 10 7081 tcttcgcattt cttttactt caccaggcgtt tctgggttagt cccaaacagg aaggcaaaaat
 7141 gcccaaaaaa agggataaag ggcacacgg aatagttagt tactcataat cttcccttt
 7201 caatattttt gaagcatttta tcagggttat tgcgtcatg gcgatcat atttgaatgt
 7261 atttagaaaaa ataaacaaat aggggttccg cgacatttc cccgaaaaagt gcccac

15

SEQ ID NO: 7 (pTnMod)

CTGACCGGCC CTGTAGCGGC GCATTAAGCG CGGGGGGTG GGTGGTTACG 50
 CGCAGCGTGA CCGCTACACT TGCCAGCGCC CTAGCGCCCG CTCCCTTCGC 100
 20 TTTCTTCCTC TCCTTCTCG CCACGTTCGC CGGCATCAGA TTGGCTATTG 150
 GCCATTGCAT ACGTTGTATC CATATCATAA TATGTACATT TATATTGGCT 200
 CATGTCCAAC ATTACCGCCA TGGTGCACATT GATTATTGAC TAGTTATTAA 250
 TAGTAATCCTA TTACGGGGTC ATTACTTCAT AGCCCATATA TGGAGTCCG 300
 CGTTACATAA CCTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC 350
 25 CCCGCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA 400
 GGGACTTTCC ATTGACGTCA ATGGGTGGAG TATTTACGGT AACTGCCA 450
 CTTGGCAGTA CATCAAGTGT ATCATATGCC AAGTACGCC CCTATTGACG 500
 TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCAGTA CATGACCTTA 550
 TGGGACTTTCTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC 600
 30 CATGGTGATG CGGTTTGGC AGTACATCAA TGGCGTGGG TAGCGGTTTG 650
 ACTCACGGGG ATTTCGAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG 700
 TTTGGCACC AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC 750
 CCCATTGACG CAAATGGGGC GTAGGGTGT ACGGTGGGAG GTCTATATAA 800
 GCAGAGCTCG TTTAGTGAAC CGTCAGATCG CCTGGAGACG CCATCCACGC 850
 35 TGTTTGACCC TCCATAGAAG ACACCGGGAC CGATCCAGCC TCCGCGGCC 900
 GGAACGGTGC ATTGGAAACGC GGATTCCCG TGCCAAGAGT GACGTAAGTA 950
 CCGCCTATAG ACTCTATAGG CACACCCCTT TGGCTCTTAT GCATGCTATA 1000
 CTGTTTTGG CTTGGGGCCT ATACACCCCCC GCTTCCCTTAT GCTATAGGTG 1050
 ATGGTATAGC TTAGCCTATA GGTGTGGGTT ATTGACCTATTGACCACT 1100
 40 CCCCTATGG TGACGATACT TTCCATTACT AATCCATAAC ATGGCTCTTT 1150
 GCCACAACTA TCTCTATTGG CTATATGCC ATACTCTGTC CTTCAGAGAC 1200
 TGACACGGAC TCTGTATTTC TACAGGATGG GGTCCCATTT ATTATTTACA 1250
 AATTCAACATA TACAACAACG CCGTCCCCCG TGCCCGCAGT TTTTATATAA 1300
 CATAGCGTGG GATCTCCACG CGAATCTCGG GTACGTGTC CGGACATGGG 1350
 45 CTCTTCTCCG GTAGCGGCCG AGCTTCCACA TCCGAGCCCT GGTCCCATGC 1400
 CTCCAGCGGC TCATGGTCGC TCGCAGCTC CTTGCTCTTA ACAGTGGAGG 1450
 CCAGACTTAG GCACAGCACA ATGCCACCA CCACCAAGTGT GCGCACAAG 1500
 GCGGTGGCGG TAGGGTATGT GTCTGAAAT GAGCGTGGAG ATTGGGCTCG 1550
 CACGGCTGAC GCAGATGGAA GACTTAAGGC AGCGGAGAGA GAAGATGCAG 1600
 50 GCAGCTGAGT TGGTTATTG TGATAAGAGT CAGAGGTAAC TCCCGTTGCG 1650
 GTGCTGTTAA CGGTGGAGGG CAGTGTAGTC TGAGCAGTAC TCGTTGCTGC 1700
 CGCGCGGCC ACCAGACATA ATAGCTGACA GACTAACAGA CTGTTCCCTT 1750
 CCATGGGTCT TTTCTGCACT CACCGTCGGA CCATGTGTA ACTTGATATT 1800
 TTACATGATT CTCTTACCA ATTCTGCCCTT GAATTACACT TAAAACGACT 1850
 55 CAACAGCTTA ACGTTGGCTT GCCACGCATT ACTTGACTGT AAAACTCTCA 1900
 CTCTTACCGA ACTTGGCCGT AACCTGCCAA CCAAAGCGAG AACAAAACAT 1950
 AACATCAAAC GAATCGACCG ATTGTAGGT AATCGTACCC TCCACAAAGA 2000
 GCGACTCGCT GTATACCGTT GGCGATGCTAG CTTTATCTGT TCGGGAAATAC 2050
 GATGCCATT GTACTTGTG ACTGGTCTGA TATTCGTGAG CAAAAACGAC 2100
 60 TTATGGTATT GCGAGCTCA GTCGCACTAC ACGGTCGTT TGTTACTCTT 2150
 TATGAGAAAG CGTTCCCGCT TTCAAGACAA TGTTCAAGA AAGCTCATGA 2200
 CCAATTCTCA GCCGACCTTG CGAGCATTCT ACCGAGTAAC ACCACACCGC 2250

TCATTGTCAG TGATGCTGGC TTTAAAGTGC CATGGTATAA ATCCGTTGAG 2300
 AAGCTGGTT GGTACTGGTT AAGTCGAGTA AGAGGAAAAG TACAATATGC 2350
 AGACCTAGGA GCGGAAAACCT GAAAACCTAT CAGCAACTTA CATGATATGT 2400
 5 CATCTAGTCA CTCAAAGACT TTAGGCTATA AGAGGCTGAC TAAAAGCAAT 2450
 CCAATCTCAT GCCAAATTCT ATTGTATAAA TCTCGCTCTA AAGGCCGAAA 2500
 AAATCAGCGC TCGACACCGA CTCAATTGTC CCACCCGTCAC CCTAAATCT 2550
 ACTCAGCGTC GGCAAAGGAG CCATGGGTT TAGCAACTAA CTTACCTGTT 2600
 GAAATTGCAA CACCCAAACAA ACTTGTTAAT ATCTATTGCA AGCGAATGCA 2650
 GATTGAAGAA ACCTTCGGAG ACCTGAAAAG TCCTGCTTAC GGACTAGGCC 2700
 10 TACGCCATAG CGGAACGAGC AGCTCAGAGC GTTTGATAT CATGCTGCTA 2750
 ATCGCCCTGA TGCTTCAACT AACATGTTGG CTTGCGGGCG TTCATGCTCA 2800
 GAAACAAGGT TGGGACAAGC ACTTCCAGGC TAACACAGTC AGAAATCGAA 2850
 ACGTACTCTC AACAGTCGC TTAGGCATGG AAGTTTGCG GCATTCTGGC 2900
 TACACAATAA CAAGGGAAGA CTTACTCGTG GCTGCAACCC TACTAGCTCA 2950
 15 AAATTTATTC ACACATGGT ACGCTTTGGG GAAATTATGAA TAATGATCCA 3000
 GATCACTCTC GGCTAAATAA AGATCAGAGC TCTAGAGATC TGTTGTTG 3050
 TTTTTGTTGG ATCTGCTGT CTTCTAGTT GCCAGCCATC TGTTGTTGC 3100
 CCCCTCCCCG TGCCCTCTT GACCTCTGGAA GGTGCCACTC CCACTGCTCT 3150
 TTCCATAATAA AATGAGGAAA TTGCATGCA TTGCTGAGT AGGTGTCATT 3200
 20 CTATTCTGGG GGGTGGGGTG GGGCAGCACA GCAAGGGGGA GGATTGGGAA 3250
 GACAATAGCA GGCATGCTGG GGATGCGGTG GGCTCTATGG GTACCTCTCT 3300
 CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT 3350
 CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CCGTACCAAGG TGCTGAAGAA 3400
 TTGACCCGGT GACCAAGGT GCCTTTTATC ATCACTTTAA AAATAAAAAA 3450
 25 CAATTACTCA GTGCCGTGTA TAAGCAGCAA TTAAATTATGAA TTGATGCCA 3500
 CATCACAAACA AAAACTGATT TAACAAATGG TTGGTCTGCC TTAGAAAGTA 3550
 TATTGAAACA TTATCTTGTAT TATATTATTG ATAATAATAA AAACCTTATC 3600
 CCTATCCAAG AAGTGATGCC TATCATTGGT TCGAATGAAC TTGAAAAAAA 3650
 TTAGCCTGTA ATACATTACT GGTAAAGGTAAC CGCCATTGT CAGCAAATTG 3700
 30 ATCCAAGAGA ACCAACTTAA AGCTTCTCG ACAGGAATGTT AATTCTCGTT 3750
 GACCCGTGAGC ACTGATGAAT CCCCTAATGA TTTTGGTAA AATCATTAAG 3800
 TTAAAGTGGG TACACATCTT GTCATATGAT CCCGGTAATG TGAGTTAGCT 3850
 CACTCATAG GCACCCCGG CTTTACACTT TATGCTTCCG GCTCGTATGT 3900
 TGTGTGGAAAT TGTGAGCGGA TAACAAATTTC ACACAGGAAA CAGCTATGAC 3950
 35 CATGATTACG CCAAGCGCGC AATTAACCCCT CACTAAAGGG AACAAAAGCT 4000
 GGAGCTCCAC CGCGGTGGCG GCCGCTCTAG AACTAGTGGA TCCCCGGGC 4050
 TGCAGGAATT CGATATCAAG CTATCGATA CCGCTGACCT CGAGGGGGGG 4100
 CCCGGTACCC AATTGCCCCCT ATAGTGAGTC GTATTACGCG CGCTCACTGG 4150
 CCGTCCTTT ACAACGTCG GACTGGGAAA ACCCTGGCT TACCCAACTT 4200
 40 AATCGCCCTTG CAGCACATCC CCCTTCGCC AGCTGGCGTA ATAGCGAAGA 4250
 GGCCCCGACC GATCGCCCTT CCAAACAGTT GCGCAGCCTG AATGGCGAAT 4300
 GGAATTGTA AGCCTTAATA TTGTTAAA ATTGCGCTA AATTTTGTT 4350
 AAATCAGCTC ATTTTTAAC CAAATAGGCCG AAATCGGCAA AATCCCTTAT 4400
 AAATCAAAAG AATAGACCGA GATAGGGTTG AGTGTGTTG CAGTTGGAA 4450
 45 CAAGAGTCCA CTATTAAGA ACCTGGACTC CAACGTCAA GGGCGAAAAA 4500
 CCGTCTATCA GGGCGATGGC CCAACTACTCC GGGATCATAT GACAAGATGT 4550
 GTATCCACCT TAATCTTATG ATTTTACCA AAATCATTAG GGGATTCTAC 4600
 AGTGTCTCAGG GTCAACGAGA ATTAACATTG CGTCAGGAAA GCTTATGATG 4650
 ATGATGTGCT TAAAAACTTA TCCATGGCT GTTGTATGCA ATCGCAATAC 4700
 50 ATGCAAAAAA CCTAAAAGAG CTGCGCGATA AAAAAGGCCA ATTTATGCT 4750
 ATTTACCGCG GCTTTTTATT GAGCTTGAAA GATAAAATAAA ATAGATAGGT 4800
 TTTATTGAA GCTAAATCTT CTTTATCGTA AAAAATGCC TCTTGGGTTA 4850
 TCAAGAGGGT CATTATATT CGCGGAATAA CATCATTGG TGACGAAATA 4900
 ACTAAGCACT TGTCTCCGT TTACTCCCT GAGCTTGAGG GTTAAACATG 4950
 55 AAGGTCACTG ATAGCAGGAT AATAATACAG TAAAACGCTA AACCAATAAT 5000
 CCAAATCCAG CCATCCAAA TTGGTAGTGA ATGATTATAA ATAACAGCAA 5050
 ACAGTAATGG GCCAATAACA CGGGTTGCACT TGGTAAGGCT CACCAATAAT 5100
 CCCTGTAAGG CACCTTGCTG ATGACTCTTT GTTGGATAG ACATCACTCC 5150
 CTGTAATGCA GGTAAGCGA TCCCACCACC AGCCAATAAA ATTAAAACAG 5200
 60 GGAAAACCTAA CCAACCTTCA GATATAAACG CTAAAAGGC AAATGCACTA 5250
 CTATCTGCAA TAAATCCGAG CAGTACTGCC GTTTTTCGC CCATTAGTG 5300

GCTATTCTTC CTGCCACAAA GGCTTGGAAAT ACTGAGTGTAAAGACCAAG 5350
 ACCCGTATG AAAAGCCAAC CATCATGCTA TTCACTCATCA CGATTCTGT 5400
 AATAGCACCA CACCGTGTG GATTGGCTAT CAATGCGCTG AAATAATAAT 5450
 5 CAACAAATGG CATCGTTAAA TAAGTGTATGT ATACCGATCA GCTTTGTTC 5500
 CCTTTAGTGA GGGTTAATTG CGCGCTTGGC GTAATCATGG TCATAGCTGT 5550
 TTCCCTGTGTG AAATTGTTAT CCGCTCACAA TTCCACACAA CATAAGGAGCC 5600
 GGAAGCATAA AGTGTAAAGC CTGGGGTGCC TAATGAGTGA GCTAACTCAC 5650
 ATTAATTGCG TTGCGCTCAC TGCCCGCTTT CCAGTCGGGA AACCTGTCGT 5700
 10 GCCAGCTGCA TTAATGAATC GGCAACCGCG CGGGGAGAGG CGGTTTGCCT 5750
 ATTGGGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTGCT 5800
 TCGGCTCGGG CGACCGGTAT CGCTCACTC AAAGCGGTAA ATACGGTTAT 5850
 CCACAGAATC AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG 5900
 CAAAAGGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT TTTTCCATAG 5950
 GCTCCGCCCC CCTGACGAGC ATCACAAAAAA TCGACGCTCA AGTCAGAGGT 6000
 15 GGCAAAACCC GACAGGACTA TAAAGATACC AGGCCTTCC CCCTGGAAGC 6050
 TCCCTCGTC GCTCTCCTGT TCCGACCCCTG CCGCTTACCG GATAACCTGTC 6100
 CGCCCTTCTC CCTCTGGGAA CGCTGGCGCT TTCTCATAGC TCACGCTGTA 6150
 GGTATCTCAAG TTGCGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC 6200
 GAAGGCGCCG TTCAGCCCGA CGCGTGCCTCC TTATCCGGTA ACTATCGTCT 6250
 20 TGAGTCCAAC CGCGTAAAGAC AGGACTTATC GCACTGGCA GCAGCCACTG 6300
 GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTCTCTG 6350
 AAGTGGTGGC CTAACACTACGG CTACACTAGA AGGACAGTAT TTGGTATCTG 6400
 CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTGAT 6450
 CGCGCAAACA AACCAACCGCT GGTAGCGGTG GTTTTTTGT TTGCAACCGAG 6500
 25 CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC 6550
 TACGGGGTCT GACGCTCAGT GGAACGAAAAA CTCACGTTAA GGGATTCTGG 6600
 TCATGAGATT ATCAAAAGG ATCTTCACCT AGATCCTTT AAATTAACAAA 6650
 TGAAGTTTA AATCAATCTA AAGTATATAT GAGTAAACCTT GGTCTGACAG 6700
 TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC 6750
 30 GTTCATCCAT AGTTGCCCTGA CTCCCCGTG TGAGATAAAC TACGATACGG 6800
 GAGGGCTTAC CATCTGGCCC CAGTGTGCA ATGATACCGC GAGACCCACG 6850
 CTCACCGGCT CCAGATTAT CAGCAATAAA CCAGCCAGCC GGAAGGGCCG 6900
 AGCCGAGAAG TGGTCCCTGCA ACTTTATCCG CCTCCATCCA GTCTATTAAAT 6950
 TGTTGCCGGG AAGCTAGACT AAGTAGTTCG CCAGTTAATA GTTGGCGCAA 7000
 35 CGTTGTTGCC ATTGCTACAG GCATCGTGGT GTCACGCTCG TCGTTGGTA 7050
 TGGCTTCATT CAGCTCCGGT TCCCAACGAT CAAGGGCAGT TACATGATCC 7100
 CCCATGTTGT GCAAAAAAGC GGTTAGCTCC TTGGTCCCTC CGATCGTTGT 7150
 CAGAAGTAAG TTGGCCCGAG TGTTATCACT CATGGTTATG GCAGCACTGC 7200
 ATAATTCTCT TACTGTCATG CCATCCGTAA GATGTTTTC TGTGACTGGT 7250
 40 GAGTACTCAA CCAAGTCATT CTGAGAAATAG TGATGCGGC GACCGAATTG 7300
 CTCTTGCCCG GCGTCATAAC GGGATAATAC CGCGCCACAT AGCAGAACTT 7350
 TAAAAGTGTCT CATCATGGAA AACGTTCTT CGGGGCGAAA ACTCTCAAGG 7400
 ATCTTACCGC TGTTGAGATC CAGTTCGATG TAACCCACTC GTGCACCCAA 7450
 CTGATCTCA GCATCTTTA CTTCACCAAG CGTTTCTGGG TGAGCAAAAAA 7500
 45 CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC ACGGAAATGT 7550
 TGAATACTCA TACTCTTCCT TTTCAATAT TATTGAAGCA TTTATCAGGG 7600
 TTATTGTCTC ATGAGCGGAT ACATATTGAA ATGTATTAG AAAAATAAAC 7650
 AAATAGGGGT TCCGCGCACA TTTCGGAA AAGTGCCAC 7689

50 SEQ ID NO:8 (modified Kozak sequence)
ACCATG

SEQ ID NO:9 (a Kozak sequence)
ACCATGG

55 SEQ ID NO:10 (a Kozak sequence)
ACCATGT

60 SEQ ID NO:11 (a Kozak sequence)
AAGATGT

SEQ ID NO:12 (a Kozak sequence)
ACGATGA

5 SEQ ID NO:13 (a Kozak sequence)
AAGATGG

10 SEQ ID NO:14 (a Kozak sequence)
GACATGA

15 SEQ ID NO:15 (a Kozak sequence)
ACCATGA

20 SEQ ID NO:16 (a Kozak sequence)
ACCATGT

25 SEQ ID NO:17 (conalbumin polyA)
tctgccattg ctgcttcctc tgcccttcct cgtcaactcg aatgtggctt cttcgctact
gccacagcaa gaaaataat ctaaacatct aataatgggtt cctgagggtt ttcaagagtc
gttaagcaca ttcctccccc agcacccctt gctgcaggcc agtgcaggc accaaacttgg
30 ctactgctgc ccatgagaga aatccagttc aataatttcc aaagcaaaaat ggattacata
tgccctagat cctgattaac aggcgttgtt attatctagt gctttcgctt caccaggatt
atccccattgc ctccc

35 SEQ ID NO:18 (synthetic polyA)
GGCGCCCTGGATCCAGATCACTCTGGCTAATAAAAGATCAGAGCTCTAGAGATCTGTGTGTTGGTTTT
TGTGGATCTGCTGTGCCCTCTAGTTGCCAGCCATCTGTTGTTGCCCTCCCCGTGCCCTTGTGACC
CTGGAAGGTGCCACTCCACTGTCCTTCCTAATAAAATGAGGAAATTGCATCGCATTGCTGAGTAGG
TGTCAATTCTATTCTGGGGGGTGGGGTGGGGCAGCACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGG
CATGCTGGGATGCCGTGGCTCATGGGTACCT
40 SEQ ID NO:19 (avian optimized polyA)
ggggatcgc tctagagcga tcgggatct cggggaaagc gttgggtgacc aaaggtgcct
tttatcatca cttttaaaat aaaaaacaat tactcagtc ctgttataag cagcaattaa
45 ttatgattga tgcctacatc acaaaaaaa ctgatttaac aatgtttgg tctgccttag
aaagtatatt tgaacattat cttgattata ttattgataa taataaaaac cttatcccta
tccaagaagt gatgcctatc attgggtgga atgaacttga aaaaatttag ctttgaatac
attactggta agttaaacgc cattgtcagc aaattgtatcc aagagaacca a

50 SEQ ID NO:20
(vitellogenin promoter)
TGAATGTGTT CTTGTGTTAT CAATATAAAT CACAGTTAGT GATGAAGTTG GCTGCAAGCC
TGCATCAGTT CAGCTACTTG GCTGCATTGTT GTATTTGGTT CTGTAGGAAA TGCAAAAGGT
55 TCTAGGCTGA CCTGCACTTC TATCCCTCTT GCCTTACTGC TGAGAATCTC TGCAAGTTTT
AAATTGTTCAC ATTTGCTCC CATTCTACTTT GGAAGATAAA ATATTTACAG AATGCTTATG
AAACCTTGT TCATTTAAAA ATATTCTGG TCAGCGTGAC CGGAGCTGAA AGAACACATT
GATCCCGTGA TTTCAATAAA TACATATGTT CCATATATG TTTCTCAGTA GCCTCTTAAA
TCATGTGCGT TGGTGCACAT ATGAATACAT GAATGCCAA GGTTTATCTG GATTAACGCTC
TGGCCTGCAG GAATGCCAT AAACCAAAAGC TGAGGGAAAGA GGGAGAGTAT AGTCAATGTA
60 GATTATACTG ATTGCTGATT GGGTTATTAT CAGCTAGATA ACAACTTGGG TCAGGTGCCA
GGTCAACATA ACCTGGCAA AACCAAGTCTC ATCTGTGGCA GGACCATGTA CCAGCAGGCC
GCCGTGACCC AATCTAGGAA AGCAAGTAGC ACATCAATT TAAATTATT GTAAATGCCG
TAGTAGAAGT GTTTTACTGT GATACATTGA AACTCTGGT CAATCAGAAA AAGGTTTTT
ATCAGAGATG CCAAGGTATT ATTTGATTCTT CTTTATTCGC CGTGAAGAGA ATTATGATT
GCAAAAAGAG GAGTGTTCAC ATAAACTGAT AAAAATCTG AGGAATTCAAG CAGAAAACAG
CCACGTGTTTC CTGAACATTC TTCCATATAA GTCTCACCAT GCCTGGCAGA GCCCTATTCA
CCTTCGCT

65 SEQ ID NO:21 (fragment of ovalbumin promoter - chicken)
GAGGTCAAGAT GGTTTCTTA CTGTTGTCA ATTCTATTAT TTCAATACAG
AACAAATAGCT TCTATAACTG AAATATATTG GCTATTGTAT ATTATGATTG

TCCCTCGAAC CATGAACACT CCTCCAGCTG AATTCACAA TTCCCTGTGTC
 ATCTGCCAGG CCATTAAGTT ATTCATGGAA GATCTTGAG GAACACTGCA
 AGTCATATC ATAAACACAT TTGAAATTGA GTATTGTTT GCATTGTATG
 GAGCTATGTT TTGCTGTATC CTCAGAAAAA AAGTTGTTA TAAAGCATT
 5 ACACCCATAA AAAGATAGAT TTAAATATTC CAGCTATAAGG AAAGAAAGTG
 CGTCTGCTCT TCACTCTAGT CTCAGTTGGC TCCTTCACAT GCATGCTTCT
 TTATTCCTCC TATTTGTCA AGAAAATAAT AGGTCACGTC TTGTTCTCAC
 TTATGTCCTG CCTAGCATGG CTCAGATGCA CGTTGTAGAT ACAAGAAGGA
 TCAAATGAAA CAGACTCTG GTCTGTTACT ACAACCATAG TAATAAGCAC
 10 ACTAACTAAT AATTGCTTAAT TATGTTTCC ATCTCTAAGG TTCCCACATT
 TTTCCTGTTT CTTAAAGATC CCATTATCTG GTTGTAACTG AAGCTCAATG
 GAACATGAGC AATATTTCCC AGTCTTCTCT CCCATCCAAC AGTCCGTATG
 GATTAGCAGA ACAGGCAGAA AACACATTGT TACCCAGAAT TAAAACCTAA
 TATTTGCTCT CCATTCATC CAAAATGGAC CTATTGAAAC TAAAATCTAA
 15 CCCAATCCCA TAAATGATT TCTATGGCGT CAAAGGTCAA ACTTCTGAAG
 GGAACCTGTG GGTGGGTAC AATTCAAGGCT ATATATTCCC CAGGGCTCAG

SEQ ID NO:22 (chicken ovalbumin enhancer)
 cggggctgca gaaaaatgcc aggtggacta tgaactcaca tccaaaggag
 20 cttgacctga tacctgattt tcttcaact ggggaaacaa cacaatccca caaaacagct
 cagagagaaa ccatcaactga tggtcacagc accaaggat gcaatggcaa tccattcgac
 attcatctgt gacctgagca aatgattta tctctccatg aatgggtgct tcttccctc
 atgaaaaggc aatttccaca ctcacaatat gcaacaaaga caaacagaga acaattaatg
 25 tgctccttcc taatgtcaaa attgttagtgg caaagaggag acaaataatct caagttctga
 gtaggtttta gtgattggat aaggaggat gacctgttag ctacacccgttgc cttccatatcc
 ttttgataa aaagtcttt tataacttcc aggtctccca gtctttattc atgagactgt
 tggtttaggg acagacccac aatgaaatgc ctggcatagg aaaggcagc agagccttag
 ctgacctttt cttggacaa gcattgtcaa acaatgtgtg acaaaactat ttgtactgt
 30 ttgcacagct gtgctggca gggcaatcca ttgccaccta tcccaggtaa ctttccaaact
 gcaagaagat tggcattac tctctctaga

SEQ ID NO:23 (5' untranslated region)
 GTGGATCAACATACAGCTAGAAAGCTGTATTGCTTAGCACTCAAGCTAAAAGACAACTCAGAGTTC
 ACC
 35 SEQ ID NO:24 (putative cap site)
 ACATACAGCTAG AAAGCTGTAT TGCCCTTAGC ACTCAAGCTC AAAAGACAAC TCAGAGTTCA

SEQ ID NO:25 (Chicken Ovalbumin Signal Sequence)
 40 ATG GGCTCCATCG GCCCAGCAAG CATGGAAATT TGTTTGATG TATTCAAGGA GCTCAAAGTC
 CACCATGCCA ATGAGAACAT CTCTACTGC CCCATTGCCA TCATGTGAGC TCTAGCCATG
 GTATACCTGG GTGCAAAAGA CAGCACCAGG ACACAGATAA ATAAGGTTGT TCGCTTTGAT
 AAACTTCCAG GATTCGGAGA CAGTATTGAA GCTCAGTGTG GCACATCTGT AAACGTTCAC
 TCTTCACCTA GAGACATCCT CAACCAAATC ACCAAACCAA ATGATGTTA TTGCTTCAGC
 45 CTTGCCAGTA GACTTTATGC TGAAGAGAGA TACCCAAATCC TGCCAGAATA CTTGCAGTGT
 GTGAAGGAAC TGTATAGAGG AGGCTTGGAA CCTATCAACT TTCAACACGC TGCAGATCAA
 GCCAGAGAGC TCATCAATTC CTGGGTAGAA AGTCAGACAA ATGGAATTAT CAGAAATGTC
 CTTCAGCCAA GCTCCGTGGA TTCTCAACT GCAATGGTTC TGGTTATGC CATTGCTTC
 AAAGGACTGT GGGAGAAAAC ATTAAGGAT GAAGACACAC AAGCAATGCC TTTCAGAGTG
 50 ACTGAGCAAG AAAGCAAAAC TGTGAGATG ATGTACCAAGA TTGGTTTATT TAGAGTGGCA
 TCAATGGCTT CTGAGAAAAT GAAGATCTG GAGCTTCCAT TTGCCAGTGG GACAATGAGC
 ATGTTGGTGC TGTGGCTGA TGAAGTCTCA GGCTTGAGC AGCTTGAGAG TATAATCAAC
 TTTGAAAAC TGACTGAATG GACCAGTTCT AATGTTATGG AAGAGAGGAA GATCAAAGTG
 TACTTACCTC GCATGAAGAT GGAGGAAAAA TACAACCTCA CATCTGTCTT AATGGCTATG
 55 GGCATTACTG ACGTGTGGTAG CTCTTCAGCC AATCTGTCTG GCATCTCCCTC AGCAGAGAGC
 CTGAAGATAT CTCAAGCTGT CCATGCAGCA CATGCAGAAA TCAATGAAGC AGGCAGAGAG
 GTGGTAGGGT CAGCAGAGGC TGGAGTGGAT GCTGCAAGCG TCTCTGAAGA ATTAGGGCT
 GACCATCCAT TCCTCTCTG TATCAAGCAC ATCGCAACCA ACGCCGTTCT CTTCTTTGGC
 AGATGTGTTT CCCCT

60 SEQ ID NO:26 (Chicken Ovalbumin Signal Sequence - shortened 50bp)

ATG GGCTCCATCG GCGCAGCAAG CATGGAATTT TGTTTGATG TATTCAAGGA

SEQ ID NO:27 (Chicken Ovalbumin Signal Sequence - shortened 100bp)
 ATG GGCTCCATCG GCGCAGCAAG CATGGAATTT TGTTTGATG TATTCAAGGA GCTCAAAGTC
 5 CACCATGCCA ATGAGAACAT CTTCTACTGC CCCATTGCCA

SEQ ID NO:28 (vitellogenin targeting sequence)
 ATGAGGGGGATCATACTGGCATTAGTGCTCACCCCTGTAGGCAGCCAGAAGTTGACATTGGT
 10 SEQ ID NO:29 (pro-insulin sequence)
 TTTGTGAACCAACACCTGTGCGGCTCACACCTGGGAAGCTCTCTACCTAGTGCGGGGAACGAGGC
 TTCTTCTACACACCCAAAGACCCGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGG
 15 GGCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGTCCCTGAGAACGCTGGCATTGTGGAA
 CAATGCTGTACCAGCATCTGCTCCCTCTACCACTGGAGAACTCTGCAACTAG

SEQ ID NO:30 (p146 protein)
 KYKKALKKLAKE

20 SEQ ID NO:31 (p146 coding sequence)
 AAATACAAAAAAAGCACTGAAAAAACTGGCAAAACTGCTG

SEQ ID NO:32 (spacer)
 25 (GPGG)_x

SEQ ID NO:33 (spacer)
 GPGGGPGGGPGG

30 SEQ ID NO:34 (spacer)
 GGGGSGGGGSGGGGS

SEQ ID NO:35 (spacer)
 35 GGGGSGGGGSGGGSGGGGS

SEQ ID NO:36 (repeat domain in TAG spacer sequence)
 Pro Ala Asp Asp Ala

40 SEQ ID NO:37 (TAG spacer sequence)
 Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala
 Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp

SEQ ID NO:38 (gp41 epitope)
 45 Ala Thr Thr Cys Ile Leu Lys Gly Ser Cys Gly Trp Ile Gly Leu Leu

SEQ ID NO:39 (polynucleotide sequence encoding gp41 epitope)
 Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Thr Thr Cys Ile Leu Lys Gly
 Ser Cys Gly Trp Ile Gly Leu Leu Asp Asp Asp Lys

50 SEQ ID NO:40 (enterokinase cleavage site)
 DDDDK

SEQ ID NO:41 (TAG sequence)
 Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala

55 Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Thr Thr Cys Ile
 Leu Lys Gly Ser Cys Gly Trp Ile Gly Leu Leu Asp Asp Asp Lys

SEQ ID NO:42 (altered transposase Hef forward primer)
 ATCTCGAGACCATGTGTGAACCTTGATATTTACATGATTCTCTTTACC

SEQ ID NO:43 (altered transposase Her reverse primer)
GATTGATCATTATCATAATTCCCCAAAGCGTAACC

5 SEQ ID NO: 44 GnRH:Phor 11
Met-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Lys-Phe-Ala-Ile-Cys-Lys-
Lys-Phe-Ala-Ile-Cys-OCH

10 SEQ ID NO: 45 GNRH/Phor14
EHWWSYCLPCKEAKFEAKFEAKFEAK

15 SEQ ID NO: 46 Phor14::Beta-LH Sequence
MKEFAKFAKKEFAKFAKSYAVLSCOCALCRR

SEQ	ID	NO:47 (pTnMCS (CMV-prepro-HCPro-ProLys-LC-CPA))
20	1	ctgacgcgcc ctgtagccgc gcattaagcg cggcggtgt ggtggttacg cgacgcgtga
	61	ccgtacact tgccagccgc ctacgcggc ctccttcgc ttcttcct tccttcgc
	121	ccacgttcgc cggcatcaga ttgcatttgc gccattgc acgttgc acatcatac
	181	tatgtacatt tatattggc catgttcaac attaccgcga ttgtgacatt gatttgc
	241	tagttatcaa tagtaatcaa ttacggggtc attagttcat agccatata tggagtccg
	301	cgttacataa ctacggtaa atggccggc tggctgacg ccaacgacc cccgcccatt
	361	gacgtcaata atgacgtatg ttccatcat aacgcaccaat gggacttgc attgacgtca
	421	atgggtggag tattttacgtt aaactgccc cttggcaga catcaatgtt atcatatgc
	481	aagtaacgccc cttatgtac tcaatgcgcg ttaatggccc gctgtgcatt atggccgtat
	541	catgaccta ttggacttc ctacttggca gtacatctac gtattagtca tcgcattttac
	601	catggtgatg cgggtttggc agtacatcaa tggcgttga tagcgttgc actcacgggg
	661	atttcaagt ctccacccca ttgacgtca tgggagtgg ttttgcacc aaaaatcaacg
	721	ggactttcca aatatgtcgta acaactccgc cccatgtacg ccaatggcc gttaggcgtgt
	781	acgggtggag gtctatataa cgaagactcg ttttagtgaaac cgtcagatcg cctggagac
	841	ccatccacgc tggtttgacc tccatagaa acacccggac cgatccagcc tccggggccg
	901	ggaacgggtc attggaaacgc ggattcccg tgccaagagt gacgtaaatg cccgcctata
	961	actctatagg cacacccctt tggcttattat gcatgtata ctgttttggtt cttggggcc
35	1021	atacaccatccc gtttccctat gctataggat ttatgtatc ttatgtatc ggtgtgggtt
	1081	attgaccattt attgaccact cccctatttt tgacgatattt ttccattactt aatccataac
	1141	atggctttttt gccacaacta tctctattttt ctatatgcac atactctgtc cttcagagac
	1201	tgacacggac tctgttatttt tacaggatgg ggtcccatattt attatttaca aatccataca
	1261	taacaacaacg cggccccccg tgccgcagttt tttttttaaa catagcgtgg gatctccacg
	1321	cgaatctcggtacgtgtt cggacatgggg ctcttcctcg gtagccggc agcttccaca
	1381	tccggacccctt ggtcccatgtc ctccagcgcc tcatgttgcg cttggcttca
	1441	acagtggagg ccagacttag gcacagcaca atgcccacca ccaccaatgtt gcccaca
	1501	gccgtggcg tagggatgt gtctaaaaat gaggcgtggg attgggctcg cacggctgac
	1561	gcagatggaa gacttaaggc agccggcggaa gaagatgcag gcacgttgcgt tttgttattt
	1621	tgtataagat cagaggtaac tcccggttgc gtgctgttac cgggtgggg cagtgtatgc
	1681	tgagcgtact tctgttgcgc cggcgcgc accagacata atagctgaca gactacaca
	1741	ctgttccctt ccatgggtct tttctgcagt caccgtcgga ccatgtcgaa actcgatatt
	1801	ttacacgact ctctttacca attctgcccc gaattacact taaaacgact caacagctt
	1861	acgttggctt ggcacgcattt acttgcattt aaaaactctt ctcttaccca acttggccgt
	1921	aacctggccaa ccaaaacgcgaa aaaaaacat aacatcaac gaatcgaccg atttgttagt
	1981	aatcgtcacc tccaccaaaa ggcgactcgat gtatccgtt ggcgtcgat ctttatctgt
	2041	tcgggcaata cgatgcctt tgcattttttt gactggctcg atattcgtga gaaaaaacga
	2101	ctttaggtat tgcgagcttc agtgcacta cacggcttgc ttgttactt ttatgtgaaaa
	2161	gcgttccgc tttcagagca atgttcaaa agaactctatg accaaatttc agccgaccc
	2221	gcgacatcatacggat tttttttttt caccacaccc cttatgttc gtgatgttgc cttaaagt
	2281	ccatgttata aatccgttgc gaaatgtggg tggacttgcgtt taatgtcgat aagggaaaa
	2341	gtacaatatacgttgc cttttttttt gttttttttt gttttttttt gttttttttt gttttttttt
	2401	tcatctgttc actcaaaagac tttaggtat aagaggctga cttaaagcaat tccaaatctca
	2461	tgcaccaattt tattttttttt atctcgctt aaaggccggaa aaaaatcgcc ctcgacacccg
	2521	acttgcgttgc accacccgtc acatccatccatcacttgcgttgc acatccatccatcacttgcgttgc
	2581	ctgacactaacttacttgcgttgc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
	2641	aagcgaaatgc agatttgcgc aacccatccatcacttgcgttgc acatccatccatcacttgcgttgc
	2701	ctacggccata gccgaaacggc gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
	2761	atgtttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
	2821	cacttccagg ctaacacgttgc gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
	2881	gaatgtttgc ggcattctgg tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt

5	2941	ctactagctc	aaaattttattt	cacacatgg	tacgcttgg	ggaaattttat	agggatcgca
	3001	tctagagcga	tccggatct	cgggaaaagc	gttggtgc	aaaggtgc	tttatcatca
	3061	ctttaaaaat	aaaaaaacaat	tactcagtg	ctgttataag	cagcaattaa	ttatgatttg
	3121	tgcctacatc	acaacaaaaa	ctgatattaa	aaatgttgg	tctgccttg	aaatgatata
	3181	tgaacattat	cttgatata	ttatgtatata	taataaaaaac	tttataccct	tccagaagtt
10	3241	gtatgcctatc	attgggttgg	atgaacttga	aaaaaaat	ccttgaatac	attacttgta
	3301	aggtaaacgc	cattgtcagc	aaattgtatcc	aagagaacca	acttaaaagct	ttcgcacgg
	3361	aatgttaatt	ctcggttacc	ctgagcactg	atgaatcccc	taatgattt	gttaaaaatc
	3421	attaatggtaa	ggtgatataca	catctgttca	tatgtatcc	gtaatgtgag	ttatgtcact
	3481	cattaggcac	ccccaggctt	acactttagt	cttcggctt	gtatgttgg	tggatttgt
15	3541	agcggataac	aatttccacac	aggaacacgc	tatgaccat	attacgc	ccgcgcacat
	3601	aaccctca	aaagggaaca	aaagctggag	ctccaccgc	gtggcggcc	ctctagaact
	3661	agtggatccc	ccgggctgc	ggaatccat	atcaagctt	tcgataccgc	tgacctcgag
	3721	catcagattt	gtctatggcc	attgcatac	ttgtatccat	atcataat	gtacatttat
	3781	attggctca	gttcaacatt	acccggat	tgatcatt	tattgtat	ttataatag
20	3841	taatcaatta	cggggtcatt	agttcatagc	ccatataat	agttccgcgt	tacataactt
	3901	acggtaat	gccccctgg	ctgaccgc	aacgacccc	gcccattgac	gtcaataatg
	3961	acgtatgtt	ccatagtaac	gccaatagg	actttccatt	gacgtcaat	ggtggagat
	4021	ttacggtaaa	ctgcccactt	ggcagttat	caatgtatc	atatgtca	tacccccctt
25	4081	attgacgtca	ataggctt	atggccgc	ttggcattat	cccgat	gacccattatg
	4141	gactttcctt	cttggcagta	catctacgt	ttatgtatc	ctattacat	gttgatcg
	4201	tttggcagt	acatcaatgg	gcgtggat	cgggttgc	cacgggg	tccaagtctt
	4261	cacccatgtt	acgtcattat	ggatgttgc	tggcacc	atcaacgg	ctttccaaa
	4321	tgcgttacaa	actccggcc	attgcacaa	atggcgtt	ggcgttgc	gtgggaggtc
30	4381	tatataagca	gagtcgtt	atgtacccgt	catatgc	ggagaccc	tccacgttgc
	4441	tttgaccttcc	atagaagaca	ccgggacca	tccagcc	gcggccgg	acgggtcatt
	4501	ggaaacgcgg	ttccccgtgc	caagagt	gtaatgc	cctatagact	ctataggc
	4561	acccttttgc	cttctat	tgctatact	tttttgc	ggggcctata	caccccgct
	4621	tccttatgt	ataggatgt	tgatgtatc	gcctataat	gtgggttatt	gaccattat
35	4681	gaccacttcc	cttattggta	cgatattt	ccataat	ccataacat	gtcttttgc
	4741	acaactatct	ctattggta	tatgccaata	ctctgtc	catagact	cacgactct
	4801	gtatattttac	aggatgggt	cccatattt	attacaaat	tcacatatac	aacaacgc
	4861	tcccccgtgc	cccgatgtt	tat	aaacat	agegttgc	ctccacgc
	4921	cgtgttccgg	atagggtc	ttctcgg	gcccgg	tccacatcc	gagccctgtt
40	4981	cccatgcctc	cacgggtca	tggcgtc	gcagtc	gtcttca	gtggaggc
	5041	gactttagca	cagcacaat	ccaccacca	ccagtgt	gcacaagg	gtggcggt
	5101	ggtagtgc	tggaaat	cgtaggat	gggtcgc	ggtagcgc	gatggaaag
	5161	ttaaggc	ggcagaaag	gatgcagg	gctgatgt	tgtatct	taagatgt
	5221	aggtacttcc	cgttgcgt	ctgttac	ttggggc	tgatgtc	gcagatct
45	5281	ttgctgcgc	gcgcgc	agacataat	gtgtac	taacagact	ttcctttca
	5341	tgggtctttt	ctgcagtc	cgtcgat	atattatc	tcgtactt	ttcgtgt
	5401	gtgttttac	atataat	at	ttttat	ttctgg	gtcaacat
	5461	atgaatttct	caaggat	tttctc	gtcgtt	gttattct	tttgc
	5521	tcggctgc	cagagcc	aggtaccc	gtgcgtc	aggatcc	ggggatgtt
	5581	gtaaaagg	gggggtcc	tagatct	tgtgc	ctggat	tttcagaa
50	5641	gcctggat	gtgggtcc	ccaggcttca	gggaaagg	tggatgt	cgccgtt
	5701	aaaagaaaaa	ttgtatgtt	gacaacag	tatgtc	ccgtgaa	catgttcc
	5761	atctcaagag	atgat	aaacacgtt	tatct	tgatag	cttgc
	5821	gacacagcc	tatattact	tacca	ttttat	ttttat	ttttat
	5881	cccccaattt	ggggccagg	aaccctgg	accgttct	cagccttcc	caaggccca
55	5941	tcggcttcc	cccttgc	tcctccaa	agcacctt	ggggcac	ggccctggc
	6001	tgcctgttca	aggactt	ccccaa	gtgcgg	cgtgg	acttgc
	6061	accacggcg	tgcacat	ccggcgttgc	ctacat	caggact	cttcccttgc
	6121	aacgtgttca	ccgtgc	cagcgtt	ggc	cctat	caactgt
	6181	cacaagccca	gcaacacaa	ggtggaca	aaagt	tttgc	tgacaaaact
60	6241	cacacatgc	cacccgttcc	agcacctt	gttgc	gaccgt	tttccttc
	6301	cccccaaaac	ccaaggac	cctcat	tccggat	cc	gaggat
	6361	gttgcgttca	ggcagaa	ccctg	ggat	tttgc	atgcgtgt
	6421	gtgcataat	ccaagacaa	ggcgcgg	gagcgt	aca	tcgtgttgc
	6481	agtccttca	ccgttcttgc	ccaggact	ctgaat	aggat	gtgcacgg
65	6541	tccacaaag	cccttcc	ccccatcg	aaaacat	ccaaat	tttgc
	6601	cgagaccc	agggttac	cgtggccca	ttccggat	agctgac	aaacccgg
	6661	aggctgac	ccctgttgc	aggcttctat	cccgac	tcggcgttgc	gtggggag
	6721	aatggc	cg	ctaca	acgc	tttgc	cgacggct
	6781	ttcttctt	acagca	cacccat	ttttat	ttttat	ttttat
	6841	tcatgttcc	tgatgt	ttttat	ttttat	ttttat	ttttat
	6901	tctccgggtt	aaagcc	ccggc	tttgc	tttgc	tttgc
	6961	gtgtcagtgt	ccccaggaca	aacggcc	atcac	tttgc	tttgc

7021 aaatatgttt attggatcca gcagaagtca gcccaggccc ctgtgggtt catcttatag
 7081 gacagcaac gaccctccgg gatcccttag agattctctg gtcaggctc agggacaatg
 7141 gccaccttga ctatcgtgg gcccagggtg gaagatgaag gtgactacta ctgttactca
 7201 actgacagca gtgggtatca tagggagggt ttacggggag ggaccaagct gaccgtccca
 7261 ggtcggccca aggctgcccc ctcgtcaact ctgttcccaac ctctctctga ggagcttcaa
 7321 gccaacaagg ccacactgtt gtgtctata agtactctt acccggggac cgtgacatgt
 7381 gcctggagg cagatacgag ccccgtaaag qccggagggtt agaccaccac accctccaa
 7441 caaagcaaca acaagtagc gcccaggcgc tacctgagcc tgacgcttga gcagtggaaag
 7501 tcccacaagg gctacatgtt ccaggtcagc catgaaggga gacccgtggaa gaagacatgt
 7561 gcccctgcag aatgttccac ggggggggg gaaaggcccccc ttttggaaagg gggggggaaac
 7621 ttcgcgcctt gactcttcgtt gtcggccat cgggggggggggggggggggggggggggggg
 7681 gccattgtt gtcctcttcgtt ctttctctgtt cactctgtt gtgggttctt tgctactgc
 7741 acagaagaa ataaaatctc aacatctaa tgggttctt gagattttc aagagtcgtt
 7801 aagcacatc cttccccggc acccccttgcgt gcaggccatg gccaggccacc aacttggctt
 7861 ctgtggccca tgagaaatcc ccgg
 7921 cctagatctt gattaacagg tttttgtat tttttgtat ttcgttccat ccacattttt
 7981 ccattgttccctgg
 8041 cgcgcgtca ctggccgtcg ttttacaacg tcgtgactgg gaaaacccctg gcggttaccca
 8101 acttaatcgc cttgcggcacc atccccctt cggcggctgg cgtatagc aagaggcccc
 8161 caccgttccgc ctttcccaaa agtggcgcag ctttgcgttca gatggggaaat tgtaagcggt
 8221 aatattttgt taaaatttgcg gtt
 8281 gccgaatcgc gaaaaatccc ttataaatca aaaaataga ccggatagg gttgagtgtt
 8341 gttccagttt ggaacaagag tccacttata aagaacgtgg actccaacgt caaagggcga
 8401 aaaacccgtt atcaggccgg tggccctt ctcggggat atatgacaag atgtgtatcc
 8461 accttttttt aatgtttttt accaaaatata ttggggattt catcgtgtt cagggtcaac
 8521 gagaatatac attccgttccat gaaatgttcat gatgtatgtt gtttttttttttttttt
 8581 ggctgggtt gcatatcgc atacatcgc aaaaacctaaa agagcttgc gataaaaaag
 8641 gccaattttt tgctattttt cggcgctttt tatttggat gaaatggaaa taaaatagat
 8701 aggtttttt tgaatggaaa ttctttttttt ctgtttttttt gtttttttttttttttttt
 8761 ggggtttttt attttcgggaa atacatcat ttgggtgcgc aataactaaat cacttgc
 8821 ctgttacttccctgttccat ggggggtttt catgggggttccatcgttccatcgttccat
 8881 acagtaaaac gctaaaccaa taatccaaat ccggccatcc ctttttttttttttttttt
 8941 ataaaataaca gcaaaacagta atggggccaa aacccgggtt gcatgggtttt ggttccat
 9001 taatccctgtt aaaggccatc gctgtatgtt cttttttttt atagacatca ctccctgtta
 9061 tgcaggtaaa gcgatccccc caccggccaa taaaatggaaa acaggggaaaa ctaaccaacc
 9121 ttccatataa aacgtttttt aacccggccaa taaaatggaaa acaggggaaaa ctaaccaacc
 9181 tggcggtttt tggccatccat gttttttttt ttttttttttttttttttttttttttttt
 9241 tgg
 9301 ctgttacttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccat
 9361 atggcatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 9421 attggcgccgtt tggcgatccatcgttccatcgttccatcgttccatcgttccatcgttccat
 9481 acaattccac acaacatccatcgttccatcgttccatcgttccatcgttccatcgttccat
 9541 gtggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 9601 tggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 9661 cgctttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 9721 gtatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 9781 aagaaatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 9841 gggtttttttt atagggtttttt ttttttttttttttttttttttttttttttttttttttt
 9901 aggtggccaa acccggccaa attt
 9961 gtggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10021 ggaaggccgtt cggccatccatcgttccatcgttccatcgttccatcgttccatcgttccat
 10081 cgccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10141 ggtttttttt ttt
 10201 actgggttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10261 tggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10321 gtt
 10381 ggttt
 10441 ctt
 10501 ttggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10561 ttt
 10621 agtggggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccat
 10681 gtcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10741 ccggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10801 gcccggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10861 cggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10921 acggccatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 10981 cgatccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgttccatcgtt
 11041 ctt

11101 ctgcataatt ctcttactgt catgccatcc gtaagatgct tttctgtac tggtagatc
 11161 tcaaccaagt cattctgaga atagtgtatg cggcgacoga gttgccttg cccggcgtca
 11221 atacgggata atacccgcgc acatagcaga actttaaaag tgctcatcat tggaaaacgt
 11281 ttcggggc gaaaactctc aaggatctt cccgtgttg gatccagttt gatgtaccc
 11341 actctgtgcac ccaactgatc tttagcatct tttacttca ccagcggtt tgggtgagca
 11401 aaaacagggaa ggcaaatgc cgcaaaaaag ggaataaagg cgacacggaa atgttgaata
 11461 ctcatactct tccttttca atattattga agcatttatac agggttattt ttcatactgac
 11521 ggatacatat ttgaatgtat ttagaaaaat aaacaataag gggttccgcg cacattcccc
 11581 cggaaagtgc cac

SEQ ID NO:48 (pTnMCS (CMV-prepro-HCPro-CPA))
 1 ctgacgcgc ctgtagcggc gcattaagcg cggcggtgt ggtggtaac cgcagcgtga
 61 cccgtactact tgccagcgcg ctagecccg ctcccttcgc ttcttcctt tcctttctcg
 121 ccaacgtcgc cggcatcaga ttggctatg gccattgtat acgtgtatc catatcataa
 181 tatgtacatt tatattggct catgtccaaat accggccaa tggacattt gatttgc
 241 tagttattaa tagtaatcaa ttacgggtc attagttcat agccatata tggatgtcc
 301 cgttacataa cttacggtaa atggcccgcc tggctgaccc cccaaacgacc cccggccatt
 361 gacgtcataa atacgtatg ttcccatag aacgccaata gggacttcc attgacgtca
 421 atgggtggag tattttacggc aaactggcc cttggcgatc catcaagtgt atcatatgc
 481 aagtagcgc cctttagcgc tcaatggcc taaatgggg gcttcgattt atgcggatc
 541 catgacccca tgggacttcc ctacttggca gtacatcttcc ttatggatca tccgtattac
 601 catgggtatg cgggttttgc agtacatcaa tgggctgttga tagcgttgg actcacgggg
 661 attccaaatg cttccacccca ttgacgtcaa tggagtttgc ttttggcacc aaatcaacg
 721 ggactttca aatgtcgta acaactccgc cccatggacg caaatggccg gtatggcggt
 781 acgggtggag gtcattataa gcaagatgtt tttatggatca cgtcaatcg cttggcgac
 841 ccatccacgc tgggttttgc tccatagaaag acacggggac cgatccagcc tccggccgg
 901 ggaacgggtc attggaaacgc ggattcccg tgccaagatg gacgtaaatg cgcctataag
 961 actctatagg cacacccctt tggcttataa gcatgtctata ctgttttgg ctggggcct
 1021 atacacccca gttccattat gctataggtt atggatgtac tttagctataa ggttgggtt
 1081 attgaccattt attgaccact cccctattttt tgacgtatc ttccattact atccataac
 1141 atggctttt gccacaacta tctctattttt ctatatggca atactctgtc ttccagagac
 1201 tgacacggac ctgtatttt tacaggatgg ggtccatattt attatttaca aattcacata
 1261 tacaacaaacg cccgtcccccc tgccgcattt tttttttttca catagcgatgg gatctccacg
 1321 cgaatctcggt acgtgttcc cggacatggg cttttctccg gtatggcgccg agctcgac
 1381 tccgacccgtt ggtccatgc tccagcgcc tcatgttgc tccggacgtc ctgttctca
 1441 acagtggagg ccagacttag gacacgacca atgcccacca ccacccgtgt gccgcacaaag
 1501 gccgtggccg tagggatgt gtttggaaat gacgtgttggg attgggctcg cacgctgtac
 1561 gcaatggaa gacttacggc acggccggaa gaaatgttgg gacgtatgtt gtttttttgc
 1621 tgataagatg cagaggtaac tcccttggc gtgtgtttaa cgggtggggg cagtgtatc
 1681 tgacgtatc tccgttgc tccggccccc accagacata atacgtacaa gactaaacaga
 1741 ctgttccattt ccatgggtct tttctgcgtt caccgtcgga ccatgtcgaa actcgatatt
 1801 ttacacgact ctcttttacca attctgcccc gaatttacact taaaacgact caacagctt
 1861 acgttgggtt gcaacgcattt atctgtactt aaaaactctca ctcttaccga acttggccgt
 1921 aacctggccaa cccaaaggcg aaaaaacat aacatcaaaat gaaatcgaccc attgttagt
 1981 aatgtcacc tccacaaaaa ggcactcgct gtataccgtt ggcgtatcg tttatgtt
 2041 tcgggcaata cgatggccat tttacttgcgtt gactggctg atattctgtg gcaaaaacgaa
 2101 ctatgtatg tgccgttcc ttttttttttgcgtt ctgttactct ttatgtggaaa
 2161 gctgttccgc tttcagagca atgttcaaaag aacatctgtt accaaatctt acggggaccc
 2221 gcgacccatc taccggatggaa caccaccccg cttatgttca gtatgttggg ctttaaatgt
 2281 ccatggtata aatccgttga gaaatgggtt tggacttggt taatgtcgatg aagggaaaa
 2341 gtacaatatg cagacccatgg aacccatccgaa gtttttttttgcgtt ttttttttttgcgtt
 2401 tcatctgtc acttcaaaacg ttttttttttgcgtt aagggatggaa ctttttttttgcgtt
 2461 tgccaaatcc tatttgcgtt atctcgctt aacccatccgaa gtttttttttgcgtt
 2521 actctatgtc accaccccgcc accttttttttgcgtt ttttttttttgcgtt
 2581 ctgacacta acttacccgtt gtttttttttgcgtt ttttttttttgcgtt
 2641 aacccatccgaa gtttttttttgcgtt ttttttttttgcgtt
 2701 ctatggccatc gccggacacgg ttttttttttgcgtt ttttttttttgcgtt
 2761 atctgttccac tttatgttgc ttttttttttgcgtt ttttttttttgcgtt
 2821 cacttccagg cttacacacgtt ttttttttttgcgtt ttttttttttgcgtt
 2881 gaagttttgc ggcatttgcgtt ttttttttttgcgtt ttttttttttgcgtt
 2941 ctatgttgc aaaaatttttccatccgaa gtttttttttgcgtt ttttttttttgcgtt
 3001 tctatggccatc ttttttttttgcgtt ttttttttttgcgtt
 3061 cttttttttttgcgtt ttttttttttgcgtt ttttttttttgcgtt
 3121 tgccatccatc aacacaaaaa ttttttttttgcgtt ttttttttttgcgtt
 3181 tgaacattat ttttttttttgcgtt ttttttttttgcgtt ttttttttttgcgtt
 3241 gatggccatc attttttttgcgtt ttttttttttgcgtt ttttttttttgcgtt
 3301 aggttaacgc ctttttttttgcgtt ttttttttttgcgtt ttttttttttgcgtt
 3361 aatgtttaat ttttttttttgcgtt ttttttttttgcgtt ttttttttttgcgtt

3421 attaagttaa ggtggataca catttgtca tatgatcccg gtaatgtgag ttagctca
 3481 cattaggcac cccaggtttt acactttatg ctccggctc gtagtggatg tggatttgt
 3541 agcggataac aatttcacac agggaaacagc tatgaccatg attacgccaa ggcgcatt
 3601 aaccctcaact aaaggaaaca aaagctggag ctccaccgcg gtggggcccg ctctagaact
 5 3661 agtggatccc cggggctgca ggaattcgat atcaagctta tgcataccgc tgaccctcgag
 3721 catcagatg gttatggcc attgcatacg ttgatccat atcataatat gtacatttat
 3781 attggctcat gtccaaacatt accggcatgt tgacattgtat tattgactat ttatataat
 3841 taatcaatta cggggcatt agttcatagc ccataatgg agttccgcgt tacataactt
 3901 acggttaatg gccccctgg ctgaccgc aacgacccccc gcccattgac gtcaataatg
 10 3961 acgtatgttc ccatagtaac gccaataggg acttccatt gacgtcaatg ggtggagtt
 4021 ttacggtaaa ctgcccattt ggcgatcatc caatgttcat atatgtcaag tacgccccct
 4081 attgactca atgacggtaa atggccgcg tggcattatg cccagttacat gaccttatgg
 4141 gactttctca ttggcagta catctacgtt attagtcatcg ctattaccat ggtgatgcgg
 4201 ttttggcagt acatcaatgg gctgttgcgatg cgttgcgttact caccgggatt tccaagtctt
 15 4261 cacccttgg acgtcaatgg gaggatgtt tggcaccataa tcaacggga ctttccaaaa
 4321 tgcgttaaca actccggccc attgacgcaaa atggccggta ggcgtgtacg gtggggagtc
 4381 tatataagca gagctcgatggt agtgaaccgtt cagatcgctt ggagacgcca tccacgtgt
 4441 tttgacccca atagaagaca cggggaccga tccagcctcc gcccggggaa acgggtgcatt
 4501 ggaacccggaa ttcccccgtc caaggtgac gtaatgttccctt ctttggcttata caccggcgt
 20 4561 acccccttgg atctttatgcg tgcataacttgg ttttggcttata caccggcgt
 4621 tccttatgtt ataggatgtt gtagtgcataa gctttaggtt gttgggttattt gaccattatt
 4681 gaccactccc ctattggta cgataacttgc cattactaat ccataacatg gctctttgc
 4741 acaactatctt ctattggta tatggcaata ctctgtcctt cagagactga cacggactct
 4801 gtatttttac aggtgggggtt cccattttt attacaaat ttcacatataa aacaacccgg
 25 4861 tccccctgg cgcgcgtt tattaaatcat gtcgtggggat ctccacgcga atctcggtt
 4921 cgtgtccgg acatgggtt tttccggta gggccggagc ttccacatcc gggccctgg
 4981 cccatgcctc cagcgcgtca tggcgcgtc gcaacttgcgtt gtcctaaaca gtggaggcca
 5041 gacttaggca cagcacatgg cccaccacca ccagtgtcc gcaacaggcc gtggcggttag
 5101 ggtatgttc tgaaaatggc cgtggatgg ggcgcgtc ggcgtacgca gatggaaagac
 5161 ttaaggcagc ggcagaagaa gatgcggca gctggatgtt tgatattctga taagagtca
 5221 aggttaactcc cgttgcgtt ctgttaacgg tggagggcag tggatgttca gcaacttgc
 5281 ttgctgcgcg ggcgcggacc agacataataa gtcgtacagac taacagactg ttcccttcca
 5341 tgggtctttt ctgcgtcact cgttgcgttca atccatcgc tgcgtactt ttcgtgttgc
 5401 gtgtttactt atataatcaa atttatattt tgcgttattt aaatttataa tatttcgac
 35 5461 atgaattttt ctggatattt tttcttcgtt ttcgtttttt ttctggctt gtcacaaatgt
 5521 tcggctgcgc cagaggccaa aggtacccag gtcgcgtc aggacttgcg gggaggcttgc
 5581 gtaaaaggccgg gggggccctt tagacttcc tgcgtcgcgtt ctggatttccat ttccagaac
 5641 gcttggatggc gcttggccgg ccaggcttccca gggaaaggggc tggatgtgggtt cggccgttatt
 5701 aaaaaggccaaa ttgtatgttgg gacaacagac tattgttgcac cctgttgcac gaaaggccgg
 40 5761 attcctaaatgg atgatggggaa aacacgttta tattgttgcac tttttttttt gaaaggccgg
 5821 gacacaggccg tatattacttgc taccacgggg attatgttgcac cttttttttt gttttttt
 5881 ccccccattt gggggccaggg aaccctgttcc accgttccctt caccgttccac caaggcccca
 5941 tccgttccctt ccctgttccac tcccttccaaag agcacccttgc gggccacagg gggccctgg
 45 6001 tgcgttccctt ccctgttccac tcccttccaaag agcacccttgc gggccacagg gggccctgg
 6061 accagcgccg tgcacacccctt tccgttccctt ctacagttccctt caccgttccac tttcccttgc
 6121 aacgtgttgc cctgttccctt caccgttccac tcccttccaaag agcacccttgc gggccacagg
 6181 cacaaggccca gcaacaccaa ggtggacaaag aatgttgcac ccaatgttgc gggccacagg
 6241 cacacgttccca caccgttccca gggccacagg tcccttccaaag gggccacagg gggccacagg
 6301 ccccaaaatcccaaggccac cccatgttccca tcccttccaaag gggccacagg gggccacagg
 6361 gtggacgttgc gccacgaa gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 6421 gtgcataatg ccaagacaaa gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 6481 agcgttccctt ccctgttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 6541 tccaaacaaatcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 6601 cggacccacccatcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 6661 agcgttcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 6721 aatggggcagc cggacccacccatcccttccca gggccacaggccac tcccttccaaag gggccacagg
 6781 agcgttcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 6841 tccatgttcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 6901 tcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 6961 tcgttcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 7021 tatgtttttt ggttcccttccca gggccacaggccac tcccttccaaag gggccacagg
 7081 agcgttcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 7141 accttgcataatg cccatgttccca gggccacaggccac tcccttccaaag gggccacagg
 7201 gacacgttcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 7261 cggacccacccatcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg
 7321 aacaaggccca cactgttcccttccca gggccacaggccac tcccttccaaag gggccacagg
 7381 tggaggccca atagcggccca cggacccatcccttccca gggccacaggccac tcccttccaaacaa
 7441 agcgttcccttccca gggccacaggccac tcccttccaaag gggccacagg gggccacagg

7501 cacaaaagct acagctgccs ggtcacgcata gaaggggagca ccgtggagaa gacagtggcc
 7561 cctgcagaat gttcacccgcg gaggggaggga agggcccttt ttgaaggqgg aggaaacttc
 7621 gcgccatgac tcctctcgta ccccccgcac ggaacactga tgcgcagagg gcccctcgcc
 7681 attgcgtctt cctctgcctt tcctcgta ctcgaatgtg gctttttgc tactgcccaca
 5 7741 gcaagaataa aaatctcaac atctaaatgg gttccctgag atttttcaag agtcgttaag
 7801 cacattcctt ccccagcacc cttctgcga ggccagtgcc aggcaccaac ttggctactg
 7861 ctgcccata gagaatcca gttcaatatt ttccaaagca aaatggatta catatgcct
 7921 agatcctgat taacagggt tttgtattat ctgtcttc gttcaccca cattatccca
 7981 ttgcctccccc tcgagggggg gcccggtacc caattcgccc tatagtgagt cgtattacgc
 10 8041 gcgctcaactg gccgtcgat tacaacgtcg tgactggaa aaccctggcg ttacccaact
 8101 taatccctt gcagccatc ccccttcgc cagtcggct aatagcgaag agggccgcac
 8161 cgatcgccct tcccaacagt tgccgacgct gaatggcga tggaaattgt aagcgttaat
 8221 attttttaa aattcgcgtt aaattttgt taaatcagct catttttaa ccaataggcc
 8281 gaaatccgca aaatccctt taaatcaaaa gaatagaccc agatagggtt gagtggtt
 8341 ccagtggta aacagatcc actatcaag aacgtggact ccaacgtcaa agggcgaaaa
 8401 accgtctatc agggcgttgc cccactactc cgggatcatc tgacaagatg tgcattccacc
 8461 ttaacttaat gattttacc aaatcattt ggggattcat cagtgcgt ggtcaacgag
 8521 aattaaacatt ccgtcaggaa agcttatgtat gatgtatgtc taaaaaactt actcaatggc
 8581 ttgttatgca ttcgcata catcgaaaa acctaaaaa gcttgcgtat aaaaaaggcc
 8641 aatttttgc tatttccgc ggcttttat tgagcttga agataaataa aatagataagg
 8701 ttttttttga agcttaatct tctttatcgt aaaaatggc ctcttgggtt atcaagagg
 8761 tcattatatt tcgccaata acatcattt gtagccaaat aactaagcac ttgtctccgt
 8821 ttactccccc tgagggttag gggtaacat gaaggatcat gatagcagga taataataca
 8881 gtaaaacgtt aaccaataaa tccaaatcca gccatccaa atttgtatg aatgattata
 8941 aataacacca aacagatatc ggcataaacc accgggttgc tggtaaggc tcaccaataa
 9001 tccctgtaaa gcacccgtat gatgtactt tttttggata gacatactc cctgtatatc
 9061 aggttaaagcg atccaccac cagccaataa aattaaaaca gggaaaacta accaaccttc
 9121 agataaaaac gctaaaaagg caaatgcact actatgcata ataaatccga gcagactgc
 9181 cgtttttcg cccattatgg gcttattttt cctgcaccaaa aggcttggg tactgagtgt
 9241 aaaaagacca gaccgttaat gaaaagccaa cccatgtctt atccatcatc acgatttctg
 9301 taatagcacc acaccgtgtc ggattggctt tcaatgcgtt gaaataataa tcaacaaatg
 9361 gcatcgtaa ataatgtatg tataccgatc agcttttttgc ccttttagt agggtaatt
 9421 ggcgcgttgc cgtatcatg gtcatacgatg ttccgtgtt gaaatttttgc tccgtcaca
 9481 attccacca acatacgaccc cggaaagcata aatgtttaag cttgggtgc ctaatgatgt
 9541 agctaactca cattatgtc ttgcgcgtca ctgcggcgtt tccagtcggg aaacctgtcg
 9601 tgccagtcgatc attaatgtatc cggccaaacgc ggggggggg ggggtttgc tattgggcgc
 9661 tcttccgtt ctcgcgtcact tgatcgatc cgtcggtcg ttcgggtcg gcgagcggtt
 9721 tcagtcact caaaggcggtt aatacgggta tccacggataa cggggatataa cgcaggaaag
 9781 aacatgttag cttttccata gggccccc ccctgacgatc catcacaatc atcgcgttgc aatgtcgagg
 40 9841 tttttccata ggtccccc ccctgacgatc catcacaatc atcgcgttgc aatgtcgagg
 9901 tggcgaaacc cggccggact ataaagatatac cggccgttcc cccctggaaat ctcctcgat
 9961 cgcgttgc ttcgcggaccat ggcgttacc ggtatctgtt ccccttgc ccccttggaaat
 10021 agcgtggcgc ttctctatag ctcacgtgtt aggtatcttc gtcgggtgtt ggtcggtcg
 10081 tccaaatgtt ggttttttgc gcaaaaaaaa gtcacccccc gtcacccccc accgcgtgcgc cttatccgtt
 10141 aactatcgatc ttgatgttcaaa cccgttaaga caccgttataat cgcacttgc agcagccact
 10201 ggttaacggaa tttagcggatc gggatgttgc ggcgttgcata cagatgtttt gaaatgggtt
 10261 cctaaatcgatc gtcacatcgatc aaggatgttgc tttttatctgc ggcgttgc gaaaggccat
 10321 accttcggaa aaaaatgttgc ttttttttttgc tccggccaaac aaccacccgc tggtagcggt
 10381 ggttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 10441 ttgatctttt ctacgggttgc tgacgtcgat gtttttttttgc ttttttttttgc ttttttttttgc
 10501 gtcatcgatc tatcaaaaatg gatcttcacc tagatctttt ttttttttttgc ttttttttttgc
 10561 aatcaatcgatc aaaaatgttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 10621 gaggcaccatc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 10681 gtgtatataa ctacgtatcgatc ggggggttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 10741 cggccggccatc gtcacccccc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 10801 gggccggccatc gtcacccccc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 10861 gggccggccatc gtcacccccc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 10921 gggccggccatc gtcacccccc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 10981 tcaaggcgatc ttacatgtatc cccatgttgc ttttttttttgc ttttttttttgc ttttttttttgc
 11041 cccatgtatc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 11101 ctttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 11161 accaaatcgatc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 11221 cggccggccatc gtcacccccc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 11281 ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 11341 ctttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 11401 acggccggccatc gtcacccccc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 11461 atacttcctt ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc
 11521 tacatatttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc ttttttttttgc

11581 aaagtgcac

SEQ ID NO: 49 pTnMCS (Chicken OVep+OVg'+ENT+proina+syn polyA)

5 1 ctgacgcgcc ctgttagccgc gcattaaagcg cggccgggtgt ggtggttacg cgcagcgtga
 61 ccgctacact tgccagccgc ctacgcggc ctccttcgc ttcttcctt tccttcgc
 121 ccacgttcgc cggcatcaga ttggctattt gccattgcatt acgttgtatc catabataa
 181 tagtgtacatt tatattggct catgttcaac attacccca tggatgtacatt gattattgac
 241 tagtattaa tagtaatcaa ttacggggc attagttcat agccatataa tggatgtccg
 10 301 cgttacataa cttacgttaa atggccgc tggctgaccg cccaaacgacc cccgcccatt
 361 gacgtcaata atgacgtatg tttccatagt aacgcaataa ggacttcc attgacgtca
 421 atgggtggag tatttacggt aaactgccc cttggcagta catcaagtgt atcatatgca
 481 aagtacgccc cttattgacg tcaatgacgg taaatggccc gcttgcattt atgcccagg
 541 catgacccat tgggacttc ctactggc atcacatcgtt gatattgtca tcgcttattac
 15 601 catgttgatc ctttttttgc agtacataa tggctggta tagcgggtt actcacgggg
 661 atttcaatg ctccacccca ttgtacgttca tggaggtttt tttggacc aaaaatcaacg
 721 ggactttcaaa aatgtcgta acaactccgc cccattgacg caaatggcg gtaggcgtgt
 781 acgggtggag gtctatataa gcagagctcg ttttagtgaac cgtcagatcg ccttgagacg
 841 ccatccacgc tggttttgc tccatagaag acacccggac cgttccagcc tccgcggccg
 20 901 ggaacgggtgc attggaaacgc ggatccccc tgccaaaggt gacgtaatg cccctatag
 961 actctatagg cacacccctt tggctttat gcatgttataa ctgttttttgg ctggggcc
 1021 atacacccca gttttttat gctataggtt atgttataa ttggcttataa ggtgtgggtt
 1081 atggaccattt attggaccattt cccctattttt tgacgataactt ttccattactt aatccataa
 1141 atggctttt gccacaaactt tcttgcattt ctatgttca atactctgtc ttccagagac
 1201 tgacacggac tctgtttttt ttcacggatgg ggtttttttt aatttttaca aattttcaca
 1261 tacaacaacg cccgtcccccg tgccgcgtt tttttttttt aatagcgtt gatctccacg
 1321 cgaatctcggtt gtagtgcggc cggacatggg ctcttcccg gtagcgggg agtttccaca
 1381 tccgagccctt ggtttccatg ctccacggc tcacggcgtt cttggcgtt cttggcttca
 1441 acagtggagg ccacatggcc acatggccatc atggccacca ccacccgtt gccgcacaag
 1501 gccgtggccg taggttatgt gtcgtttttt gtcgtttttt gtcgtttttt ctcgcgtac
 1561 gcacatggaa gacttaaggc agcggcggaa gaagatgcag gcaacgtt gttgttattc
 1621 tgataaggtt cagaggttac tcccttgcgtt gtcgtttttt gtcgtttttt ctcgcgtac
 1681 tgacgttac tccgttgcgtt cccggccggcc accacacata atagctgaca gactaacaga
 1741 ctgttccatc ccatgggtt ttttcgtt caccgttgcgc ccatgttgcgc actcgatatt
 1801 ttacacgtt ctcttttacca attcttgcctt gattttttt aaaaacactt caacagctt
 1861 acgttggctt gccacgcattt acttgcactt aaaaacttca ctcttaccga acttggccgt
 1921 aacctgccaa ccaacggcggaa aacaaaacat aacatcaacaaat gaaatcgaccc attgtttaggt
 1981 aatcgtaacc cccatggatgtt gtcgtttttt gtcgtttttt gtcgtttttt cttttatctgt
 2041 tcggcaataa ccatggccat ttttcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2101 ctatgttat ttcacggctt acttcgttca ctcacggcgtt ctgttactt ttatgagaaa
 2161 gctgtttccgc ttttcacggatgtt atgttcaatggg aacatgttcatg accaattttt agccgaccc
 2221 gctgtttccgc ttttcacggatgtt atgttcaatggg aacatgttcatg accaattttt agccgaccc
 2281 ccatggatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2341 ttcacatgtt cccatggatgtt atgttcaatggg aacatgttcatg accaattttt agccgaccc
 2401 tcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2461 tgccaaatcc ttttcacggatgtt atgttcaatggg aacatgttcatg accaattttt agccgaccc
 2521 acttcattgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2581 ttcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2641 aacgttgcgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2701 ctacgcgcataa gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2761 atgttgcgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2821 ctcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2881 gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 2941 ctatgttgcgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3001 ttcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3061 ttcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3121 ttcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3181 ttcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3241 gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3301 aggttgcgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3361 aatccgttgcgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3421 attcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3481 ctcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3541 aggttgcgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3601 aaccccttactt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3661 aggttgcgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3721 ttcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt
 3781 ctcacatgtt aatccgttgcgtt gtcgtttttt gtcgtttttt gtcgtttttt gtcgtttttt

3841 attcatctgt gacctgagca aatgattta tctctccatg aatgggtgct tctttccctc
 3901 atgaaaaggc aatttccaca ctacaatata gcaacaaaga caaacagaga acaattaatg
 3961 tgctccctcc taatgtcaaa attgtatgg acaagaggag acaaaaatct caagtctg
 4021 gtaggtttt gtgatggat aagaggctt gacctgtgag ctcacctgga cttcatatcc
 4081 ttttggataa aaagtgcattataaatttc aggtctccg gtctttatc atgagactgt
 4141 tgggttaggg acagacccac aatgaaatgc ctggcatagg aagggcagc agagccttag
 4201 ctgacccctt cttggacaa gcattgtcaa acaatgtgtg acaaaaactat ttgtactg
 4261 ttgcacactgt gtcgtggca gggcaatcca ttgcaccta tcccaaggta cttccaact
 4321 gcaagaagat tggcttac tctctctaga aagttctgc agactgacat gcatatccata
 4381 ggttagagata acattactg ggaacgacat ctatcatcat aaaaaggcagg caagattttc
 4441 agactttt agtggctgaa atagaagcaa aagacgtgat taaaacaaa atgaaacaaa
 4501 aaaaatcatgt tgatccatgt ggttagaca tccagcaaa aatattatc tgcactacca
 4561 tcttgcattt agtccatcaga cttggcaagg agaatgtaga ttctcactgt atatatgttt
 4621 tcacaaaagg aaggagagaa acaaaaggaaa atggactgta ctaaacttca gctagtgta
 4681 taggaagta attctgcattt acagagatgg cttgtatctc ttgtatgtc ctgaaattt
 4741 atgttgat tttttttttt attttttttt cttttttttt cttttttttt tttttttttt
 4801 ttcttactgt tttgtcaattt ctattatttc aatacagaac aatacgttct ataaactgaaa
 4861 tatatttgcattt attgtatattt atgattgtcc ctcgaaccat gaacacttctt ccagctgaaat
 4921 ttcacaaatc cttctgtcatc tgccaggccca ttaatgtt catggagat ctttgaggaa
 4981 cactgcaagt tcatatcata aacacatttga aatattgtt tggttttgc ttgtatggag
 5041 ctatgttttgc ttgtatcttc aaaaaaaaaaag ttgttataa agcattcaca cccataaaaaa
 5101 gatagattta aatattccatc ctataggaaa gaaagtgcgt ctgtcttca ctctgtctc
 5161 agttggccctt ttcacatcga tgcttcttta ttctccttat ttgtcaaga aaataatagg
 5221 tcacgttttgc ttctacttca tgccctgcctt agatggctc agatgcacgt tgtagataca
 5281 aagaggatca aatgaaacag acttctggc ttgttacttca accatgtttaa taagcaact
 5341 aactaaatat tgcttaattt gttttccatc tcttaagggtt ccacattttt ctgttttctt
 5401 aaagatccca ttatctgggtt gtaactgaaat ctaatggaaat catgagcaat atttcccaact
 5461 cttcttccca atccaaacatgt cttgtatggat tagcagaaca ggcggaaaaac acattgttac
 5521 cccatgttttca aactaaatattt ttgttcttca ttcaatccaa atggaccta ttgaaactaa
 5581 aatctaaatccca aatccatatttca aatgttttctt atggcgttca aagtcataact tctgaaggaa
 5641 acctgtgggtt gggtcacaat tccatgttata tattttccatc ggctcggccca gtgtatcaac
 5701 atacagctgtt aatgctgtat tgccctttagc actcaagctc aaaaagacaac tcagatgttca
 5761 ccatgggttc catcgccgc gcaacatgtt aatttttttt tgatgtt aaggagctca
 5821 aagtcaccaat tgccatgtt aacatcttctt actgtttccatc tgccatcatg tcagctctag
 5881 ccatgttataacttgc aaaaacatgttccatc aatgttccatc aatgttccatc aatgttccatc
 5941 ttgataaacttccatc aatgttccatc aatgttccatc aatgttccatc aatgttccatc
 6001 ttcaacttccatc aatgttccatc aatgttccatc aatgttccatc aatgttccatc
 6061 tcacgttttgc cttgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6121 agtggactgtt aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6181 atcaagccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6241 atgttccatc gcaacatgtt aatgttgc aatgttgc aatgttgc aatgttgc
 6301 tcttcaaaagg aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6361 gagtgactgtt gcaaggaaaggc aatgttgc aatgttgc aatgttgc aatgttgc
 6421 tggcatcaat ggttttgc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6481 tgagcatgtt ggttgcgtt cttgtatgtt aatgttgc aatgttgc aatgttgc
 6541 tcaacttgc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6601 aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6661 cttatgttgc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6721 agggccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6781 gagggatgtt aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6841 gggccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6901 ttggcatgtt ggttttgc aatgttgc aatgttgc aatgttgc aatgttgc
 6961 atggccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7021 gtatccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7081 accaaacatgtt gtcgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7141 gcttccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7201 agtggccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7261 agaaggcttgc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7321 actactgttgc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7381 ggttccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7441 ttggcatgtt gtcgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7501 cttccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7561 catgttgc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7621 agggggatgtt ggttttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7681 cttccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7741 gggccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7801 ttggcatgtt gtcgttgc aatgttgc aatgttgc aatgttgc aatgttgc
 7861 tccatc aatgttgc aatgttgc aatgttgc aatgttgc aatgttgc

7921 gttgcgcagc ctgaatggcg aatggaaatt gtaagcgta atatttgtt aaaattcgcg
 7981 tttaatttt gttaaatcg ctcattttt aaccaatagg ccgaatcg caaaatccct
 8041 tataaatcaa aagaatagac cgagataggg ttgagtgtt ttcgcgttgg gaacaagagt
 8101 ccactattaa agaacgtgga ctccaaacgtc aaaggcgaa aaacccgtcta tcagggcgat
 8161 ggccactac tccggatca tatgacaaga tttgtatcca ctttaactta atgatttta
 8221 cccaaatcat tagggatc atcagtgctc aggtcaacg agaattaaca ttccgtcagg
 8281 aaagttatg atgatgtatg gttttttttt tttttttttt tttttttttt tttttttttt
 8341 tacatgcgaa aaacctaaaaa gagcttgcgg ataaaaaagg ccaatttattt gttttttt
 8401 gccgtttttt attgaggtt aaagatataat aaaaatagata gttttttttt gttttttt
 8461 cttcttttac gttttttttt ttcccttggg ttatcaagag gtttattttttt tttcccttgg
 8521 taacatcatttggtgacgaa ataaactaaacg atttgtctc ttgttactcc ctttgcgtt
 8581 aggggttaac atgaaggctca tcgatagcag gataataataa cagttttttt cttttttt
 8641 aatccaaatc cagccatccc aaatttggtag tgaatgatta taaaataacag cttttttt
 8701 tggccaaata acacccgggtt cttttttttt tttttttttt tttttttttt tttttttttt
 8761 ctgtatgc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 8821 accagccaaatc aaaaataaaaa cgggggggggggggggggggggggggggggggggggggg
 8881 ggcaatgccttt
 8941 gtggcttatttcccttcccttcccttcccttcccttcccttcccttcccttcccttcccttccctt
 9001 atgaaaaaggcc aaccatcatc ctattttttttt tttttttttt tttttttttt tttttttttt
 9061 ctggatt
 9121 ttt
 9181 ttt
 9241 gccggaaagca taaagtgttaa agccgggggtt gcttaatgggggggggggggggggggggg
 9301 gccgg
 9361 atccggccaaatc ctt
 9421 actgacttcgc ttt
 9481 gtaatacgtt ttt
 9541 ctt
 9601 ccccccgg
 9661 ctatataatgg
 9721 ctggcccttt
 9781 agctcacgtt ttt
 9841 ctt
 9901 aaccgggttt
 9961 gccgg
 10021 agaaggacag ttt
 10081 ggttagcttt
 10141 ctt
 10201 ttt
 10261 aggttt
 10321 tatgatgttt
 10381 atctgtctat ttt
 10441 cgg
 10501 gctccaggat ttt
 10561 gcaacttt
 10621 tcggccatgtt ttt
 10681 tcggccatgtt ttt
 10741 ttt
 10801 aagggttt
 10861 atggccatccg ttt
 10921 tagtgcgttt
 10981 catggccatgtt ttt
 11041 aggttt
 11101 tcggccatgtt ttt
 11161 gcaaaaaagg gtt
 11221 ttt
 11281 tagaaaaataa aacaaatagg gtttttttttttttttttttttttttttttttttttt

THIS PAGE BLANK (USPTO)